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ACTA PHYSIOLOGICA SCANDINAVICA
SUPPLEMENTUM 309

THE MOLECULAR STRUCTURES OF VERTEBRATE SKIN COLLAGENS

A COMPARATIVE STUDY

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BY

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CONTENTS

SYMBOLS AND ABBREVIATIONS

I	ionic strength
T_D	denaturation temperature of solubilized collagen
T_S	shrinkage temperature
Δ	difference
$[\alpha]_\lambda$	specific rotation at wavelength λ
λ_c, λ_o	constants in Drude and Moffitt-Yang equations
TC	tropocollagen
NSC	neutral salt-soluble collagen
AC	acid-soluble or acid citrate buffer-soluble collagen
IC	insoluble collagen
SLS	segment-long-spacing form of reconstituted collagen
CM-cellulose	carboxymethylcellulose

INTRODUCTION

Mutation and natural selection are the two fundamental processes in evolution which occur at organ as well as molecular levels in living organisms. The genetic information which determines all the properties of an organism can be found in the nucleotide base sequence of its DNA which is reflected in the amino acid sequences of proteins.

Collagen is the principal protein of connective tissue. Fibroblasts synthesize the monomeric collagen unit tropocollagen and secrete it into the extracellular space where the tropocollagen molecules associate to form collagen fibrils according to a certain pattern.

The formation of connective tissue has been necessary for the development of specialized tissues and organs. Collagen is found in all animal phyla except arthropods and certain classes of coelenterata. The amount of collagen varies greatly from one species to another. In mammals collagen is the most abundant protein as it comprises about one-third of the body protein. Certain invertebrates such as horny sponges and the sea cucumber contain even higher proportions of collagen.

The purpose of the present study was to obtain information about the phylogeny of collagen and to define the essential features of collagen structure. Collagen was prepared from 12 vertebrate species chosen to represent the phylogenetic tree. These collagens were investigated by a variety of methods to obtain knowledge of the primary, secondary, tertiary and quaternary levels of collagen structure. In the interpretation of data in terms of phylogeny, care must be exercised and the following words of Florkin (1965) should be remembered: "In the present situation, we must adopt the methodical rule to be led by the knowledge of phylogeny in our search for biochemical evolution, rather than to be led by biochemistry into the discovery of new aspects of phylogeny. The Ariadne's thread of comparative biochemistry can only be the knowledge of phylogeny, in which is integrated the treasure of knowledge accumulated by generations of naturalists familiarized with living organisms."

LITERATURE REVIEW

OUTLINES OF MOLECULAR EVOLUTION

A change in a gene leading to mutation can manifest itself in several ways in DNA. Single base replacement alters the corresponding amino acid in the polypeptide chain except when the codon is degenerate. Terminal additions and deletions of amino acids, *e.g.*, in cytochrome *c* chains, have been described (Margoliash and Smith 1965). During the evolutionary development of haemoglobin, deletions are believed to have occurred because "sequence gaps" have been found in haemoglobin chains (Dixon 1966). Ingram (1961) put forward the hypothesis that the formation of new polypeptide chains in globin was a result of a complete gene duplication, which duplication may also be partial as in haptoglobin Hp2α (Dixon 1966).

As a consequence of mutation the function, which is a property of the three-dimensional structure, may remain unchanged or change over a very wide range, and even cease entirely (Margoliash 1963).

Many methods have been used in the analysis of proteins for phylogenetic purposes. The determination of the complete amino acid sequence would yield the most comprehensive information but at the moment the primary structures of only 10—15 proteins are known. Chromatography or "finger printing" of peptide fragments was used by Ingram (1958) to locate differences in various haemoglobins. Immunochemical analyses were performed by Manski, Halbert and Auerbach (1964) in their studies of the phylogeny of lens protein.

When the results of protein analyses are discussed in relation to taxonomic aspects, one must take into account the fact that only one gene has been investigated. Difficulties may also arise from the lack of homology of proteins (Florkin 1966) or from the dependence of functional similarity on convergent evolution (Mayr 1964, Zuckerkandl and Pauling 1965). Some erroneous conclusions may also be related to the translation of DNA to the amino acid sequence and *vice versa* that are due to degeneracy in the genetic code and to the non-expressed areas in the DNA sequence (Dixon 1966).

STRUCTURE OF COLLAGEN

A number of review articles (Grassmann 1960, Harkness 1961, Harrington and von Hippel 1961, Gross 1963, Harding 1964, Grassmann *et al.* 1965, Harding 1965, Zonta and Campani 1965), books (Gustavson 1956, Veis 1964, Reich 1966, review monographs edited by Hall 1963, 1964, 1965) and symposia (Stainsby 1958, Page 1959, Edds 1961, Jackson *et al.* 1965, Comte 1966) contain in summarized form the information on collagen and connective tissue published in the last ten years.

The tropocollagen macromolecule is the basic structural unit of collagen. In the native form it is a rigid rod-shaped particle 2800 Å long and 14 Å in diameter and has a molecular weight of about 300,000 as revealed by physico-chemical and electron microscopic studies of soluble collagens.

The molecule is composed of three polypeptide chains each forming a left-handed helix. The chains are further coiled into a three-stranded ropelike superhelix (triple helix). These configurations are maintained mainly by hydrogen bonds. Along the axis of the tropocollagen molecule there are sections of crystalline structure composed of nonpolar amino acids. These sections are separated by amorphous structures composed mostly of polar amino acids.

The arrangement of the chains in the tropocollagen molecule enables the formation of two kinds of collagen structure (Rich and Crick 1961). The triple helical structure presupposes that in each chain glycine is in the position nearest the axis and the pyrrolidine rings point outwards from the molecule. In collagen I the hydroxyl group of hydroxyproline is directed towards the axis whereas it points away from the axis in collagen II.

Amino acid composition

Very few analyses of amino acid compositions of collagens of different vertebrates were performed in the first half of this century. Usually one or two amino acids were determined in different gelatins. The samples were often impure and were pretreated with enzymes or alkalies that are known to modify collagen. Eastoe and Leach (1958) presented amino acid compositions of 16 different species in which they express the results in residues per 1000 residues. The effect of non-protein impurities was thus eliminated and a comparison of various proteins was possible. Reviews on amino acid compositions of collagens have been presented later by Piez and Likins (1960), Gross (1963) and Borasky (1965).

Table I presents previously reported data on amino acids of collagens of vertebrate skins. Only data obtained by ion exchange chromatography are included. They reveal, *e.g.*, that the amount of hydroxyproline and proline increases whereas that of methionine decreases in the course of evolution. The hydroxyl group content has remained constant owing to a decrease in the number of serine and threonine residues as established already by Beveridge and Lucas (1944) by rather inaccurate colour reactions.

In order to find out what is essential to collagen structure, the variation in amino acid composition was calculated (Table II). In accordance with presented models of collagen, glycine accounts for one third of the residues. The contents of acidic, basic and hydroxy amino acids are separately constant despite the great variation in the proportions of individual amino acids within these groups (see Discussion).

Table I.

Amino acid compositions of various vertebrate collagens and gelatins.

The values are numbers of amino acid residues per 1000 residues as determined by ion exchange chromatography.

	Man	Cow	Pig	Whale	Rat	Rabbit	Wallaby
Hydroxyprolines (3- & 4-)	93	90	93	89	93	102	93
Aspartic acid	46	46	46	46	45	50	49
Threonine	18	17	18	24	20	22	20
Serine	36	35	35	41	43	39	39
Glutamic acid	72	76	72	70	71	71	73
Proline	127	127	131	128	121	127	119
Glycine	327	330	328	326	331	311	320
Alanine	110	112	111	110	106	105	113
Half cystine (and cysteic acid)	<0.5	<0.5	<0.5	<0.5	—	<0.5	<0.5
Valine	25	21	24	21	24	24	23
Methionine (and sulphoxides)	6	5	5	5	8	7	7
Isoleucine	10	12	10	11	10	13	9
Leucine	25	25	24	25	24	25	26
Tyrosine	4	4	3	3	3	3	4
Phenylalanine	13	13	14	13	12	14	16
Hydroxylysines	8	6	7	6	7	5	8
Ornithine	—	<0.5	—	—	—	—	—
Lysine	25	26	26	26	27	29	25
Histidine	5	5	5	6	5	6	5
Arginine	50	50	48	50	50	47	51
Acidic amino acids	118	122	118	116	116	121	122
Basic amino acids	88	87	86	88	89	87	89
Amide N	41	43	38	26	41	40	44
Imino acids	220	217	224	217	214	229	212
Hydroxy amino acids	159	152	156	163	166	171	164
Nonpolar amino acids	643	645	647	639	636	626	631

For the occurrence of ornithine among the amino acids of collagen, the reader is referred to the articles of Hamilton and Anderson (1954), Hirs, Stein and Moore (1954) and Murray *et al.* (1965).

The numbers are average values for several specimens of collagen as follows: two *human beings* (Bornstein and Piez 1964, Eastoe 1955), eleven *cows* (Cooper and Davidson 1965, Eastoe 1955, Grassmann, Hannig and Schleyer 1960, Grassmann, Nordwig and Hörmann 1961, Hafter and Hörmann 1963, Rubin *et al.* 1965, Schlueter and Veis 1964,

Chicken	Python	Crocodile	Toad	Lungfish	Cod	Carp	Pike	Sturgeon	Shark
99	102	93	78	75	55	78	70	82	66
48	48	46	55	46	51	47	54	48	43
19	18	22	26	25	23	27	25	29	24
29	43	42	66	43	69	40	41	50	55
74	62	73	78	77	74	71	81	71	68
129	119	128	110	127	97	118	129	102	106
331	316	324	301	319	343	325	328	337	337
115	125	114	98	127	110	122	114	119	112
—	<0.5	<0.5	<0.5	<0.5	—	—	—	<0.5	<0.5
20	20	15	22	20	19	18	18	18	25
6	6	6	9	4	18	14	12	9	15
11	12	11	14	11	10	11	9	11	17
24	26	20	29	23	21	22	20	18	25
3	2	3	6	1	4	3	2	2	2
14	14	18	19	15	11	14	14	14	13
10	4	5	4	6	7	7	8	11	6
—	—	—	—	—	—	—	—	—	—
19	28	25	29	24	27	26	22	22	25
4	5	5	7	5	9	5	8	5	10
45	50	50	49	52	52	52	45	52	51
122	110	119	133	123	125	118	135	119	111
78	87	85	89	87	95	89	83	90	92
44	22	26	52	45	46	34	42	41	34
228	221	221	188	202	152	196	199	184	172
160	169	165	180	150	158	154	146	174	153
650	638	636	602	646	629	645	644	628	650

Veis and Schlueter 1964, Veis and Anesey 1965), two *pigs* (Eastoe 1955, Eastoe 1961), one *whale* (Eastoe 1955), two *rats* (Schlueter and Veis 1964), four *rabbits* (Jackson, Leach and Jacobs 1958), one *wallaby* (Eastoe 1955), one *chicken* (Leach 1957), one *python* (Leach 1957), one *crocodile* (Leach 1957), one *toad* (Leach 1957), two *lungfishes* (Eastoe 1957), two *cods* (Piez 1964, Piez 1965), two *carps* (Piez and Gross 1960, Piez, Eigner and Lewis 1963), one *pike* (Piez and Gross 1960), one *sturgeon* (Eastoe 1957), and three *sharks* (Eastoe 1957, Lewis and Piez 1964, Piez *et al.* 1963).

Table II.

Variation in the amino acid contents of various vertebrate collagens and gelatins.

The values are based on the data in Table I.

	Range	Δ	Mean	Δ/Mean
Hydroxyprolines (3- & 4-)	55—102	47	78.5	0.60
Aspartic acid	43— 55	12	49	0.24
Threonine	17— 29	12	23	0.52
Serine	29— 69	40	49	0.82
Glutamic acid	62— 81	19	71.5	0.27
Proline	97—131	34	114	0.30
Glycine	301—343	42	322	0.13
Alanine	98—127	29	112.5	0.26
Half cystine (and cysteic acid)	<0.5	—	—	—
Valine	15— 25	10	20	0.50
Methionine (and sulphoxides)	4— 18	14	11	1.27
Isoleucine	9— 17	8	13	0.62
Leucine	18— 29	11	23.5	0.47
Tyrosine	1— 6	5	3.5	1.43
Phenylalanine	11— 19	8	15	0.53
Hydroxylysines	4— 11	7	7.5	0.93
Ornithine	<0.5	—	—	—
Lysino	19— 29	10	24	0.42
Histidine	4— 10	6	7	0.86
Arginine	45— 52	7	48.5	0.14
Acidic amino acids	110—135	25	122.5	0.20
Basic amino acids	78— 95	17	86.5	0.20
Amide-N	22— 52	30	37	0.81
Imino acids	152—229	77	190.5	0.40
Hydroxy amino acids	146—180	34	163	0.21
Nonpolar amino acids	602—650	48	626	0.08

Optical properties

The total optical rotatory power of a native protein molecule depends on the amino acid composition and on the helix content of the protein. Collagen has a unique amino acid composition involving high contents of glycine and especially imino acids which makes the triple helical structure possible (Szent-Györgyi and Cohen 1957). This structure in turn is responsible for the exceptionally great levorotation of native collagen. The rotatory properties are determined by the left-handed helical charac-

Table III.

Optical properties of vertebrate tropocollagen solutions.

	Solvent	$-[\alpha]^{H}_{589}$	$-[\alpha]^{D}_{589}$	$-[\alpha]^{H}$	$-[\alpha]^{D}$	λ^{H}_{c}	λ^{D}_{c}	References
Mammals								
Man	0.15M acetate, pH 4.8	428	142	2540*	831*			1
	0.15M citrate, pH 3.7	415						2
Cow	0.15M citrate, pH 3.7	415	135					3
	"	400						4
	"			1000***	360***			5— 6
	"			1305**	468**			7
	"			1330**	460**			8
	0.1M citrate, pH 3.5	440	140					9
	0.15M acetic acid	415						10
	0.05 % acetic acid			1330**	460**	195		11—12
Rat	water	289	118			217	217	13
	citrate, pH 3.7, I 0.075	409	135					14
	"	416	133					15
	"	340	131					16
Birds								
Chick	0.5 % acetic acid	400						17
Bony fishes								
Perch	citrate, pH 3.7, I 0.075	400	128					14
	"	392	120					15
Cod	"	397	116					14
	"	380	119					15
	0.15M citrate, pH 3.0		118					18
	0.1M citrate, pH 3.6	349	107					19
	0.1M lactate, pH 3.6	403						19
	0.1M tartrate, pH 3.6	389						19
	0.1M citrate, pH 3.4	373	113					20
Eel	citrate, pH 3.7, I 0.075	398	126					14
Carp	0.15M citrate, pH 3.7					205±15		21
	citrate, pH 3.7, I 0.05	330				204		13
	0.1M acetic acid-							
	0.1M KCl, pH 2.8			1350**	460**			22
Herring	citrate, pH 3.7, I 0.075	408	125					14
Cartilaginous fishes								
Dogfish	0.15M citrate, pH 3.5	345	122					23
	"	398	124			212		24

*313 nm; **365 or 367 nm; ***405 nm

1 Bornstein and Piez (1964); *2* Bakerman (1961a); *3* Doty and Nishihara (1958); *4* Rice *et al.* (1964); *5* Kühn (1963); *6* Kühn, Fietzek and Kühn (1966); *7* Rubin *et al.* (1965); *8* Drake *et al.* (1966); *9* Steven and Tristram (1962); *10* Cooper and Davidson (1965); *11* Altgelt, Hodge and Schmitt (1961); *12* Fujimori (1966); *13* Harrington (1958); *14* Burge and Hynes (1959a); *15* Burge and Hynes (1959b); *16* Flory and Weawer (1960); *17* Levene and Gross (1959); *18* Astrup, Marko and Young (1958); *19* Young and Lorimer (1960); *20* Young and Lorimer (1961); *21* Cohen (1955); *22* Veis and Drake (1963); *23* Lewis and Piez (1961); *24* Lewis and Piez (1964).

teristics of the individual strands rather than by the right-handed superhelix formed by the three polypeptide strands (Urnes and Doty 1961). When the organized structure of native collagen is destroyed, *e.g.*, by heating, the negative rotation diminishes to a value characteristic of its amino acid composition.

Measurements of optical rotation at different wavelengths, the optical rotatory dispersion, have revealed that the dispersion by both native and denatured collagen is of a simple type and can be represented by a one-term Drude equation over the region 400—700 nm (Harrington *et al.* 1961, von Hippel and Wong 1963 b).

Table III presents values of specific rotation and optical dispersion for solutions of collagens from different species. The origin of the collagen does not seem to affect the optical properties.

Components

Denaturation of tropocollagen solutions by heat or competitors for hydrogen bonds (urea, rhodanide) destroys the organized structure. Mathews, Kulonen and Dorfman (1954) demonstrated first the separation of collagen into two components on heating its solution and ascribed the results to a reversible dissociation of procollagen monomers. These two components have been later named α and β. The β-component has twice the molecular weight of the α-component and represents a combination of two α-components. Ultracentrifugation of acid-soluble collagens yielded a component named γ which sedimented more rapidly than the β-component (Grassmann, Hannig and Engel 1961, Altgelt *et al.* 1961). Veis, Anesey and Cohen (1960) extracted a still heavier component which they named δ from bull hide corium with hot water. Molecular weight determinations revealed that the γ-component is tropocollagen, that is, a combination of three α-chains, while the δ-component is composed of four cross-linked γ-components (Veis, Anesey and Cohen 1962).

Piez *et al.* (1961) first demonstrated that purified tropocollagen from rat skin contains two types of α-chains named $\alpha 1$ and $\alpha 2$ and that it has the composition $\alpha 1$-$\alpha 1$-$\alpha 2$. The existence of a third chain type in cod skin collagen was later proved by Piez (1964, 1965) and he named the new chain $\alpha 3$. The existence of two different $\alpha 1$-chains in guinea pig collagen was proposed by Kulonen *et al.* (1965). The nonidentity of the three α-chains in rat skin and chick bone collagens has also been reported (Heidrich and Wynston 1965, Francois and Glimcher 1966).

Assuming three different α-chains there should exist three types of β-components of intramolecular origin, namely β_{12}, β_{13} and β_{23}. Up to the present only two such β-components (named β_{11} and β_{12}) have been identified, probably because of the very close chemical similarity of the $\alpha 1$- and $\alpha 3$-chains. Extraction of mammalian skin with 5 M guanidine solution has yielded a new type of component, β_{22}, which is believed to be of intermolecular origin (Bornstein, Martin and Piez 1964).

A successful separation of collagen components of equal molecular weight can at present be effected only by chromatography on carboxymethylcellulose columns (Kessler, Rosen and Levenson 1960, Piez *et al.* 1961, Schleyer 1962) or by gel electrophoresis (Näntö, Maatela and Kulonen 1963, Reich 1964, Näntö, Pikkarainen and Kulonen 1965).

Collagen structure in various vertebrates

Histology. Microscopical studies have revealed that the collagen fibres in the skins of aquatic animals are arranged side by side in laminae whereas land-living

vertebrates have a much more randomly organized pattern of collagen bundles (Gross 1963).

Molecular dimensions. Investigations of molecular weights and molecular dimensions of native tropocollagen with the aid of sedimentation analysis, light scattering measurements, viscometry and electron microscopy have not revealed any differences in the tropocollagens of calf (Doty *et al.* 1958, Engel and Beier 1963), cod (Young and Lorimer 1961), carp (Hall and Doty 1958) and spiny dogfish (Lewis *et al.* 1964).

The molecular weights of the α-, β- and γ-components do not differ from each other in man (Bornstein and Piez 1964), calf (Doty *et al.* 1958, Piez, Weiss and Lewis 1960, Grassmann *et al.* 1961a, Engel *et al.* 1963), rat (Orekhovitch and Shpikiter 1958a), cod (Piez 1965), carp (Boedtker and Doty 1956) or spiny dogfish (Lewis *et al.* 1964).

Components. Determinations of the molar ratios of the α- and β-components in soluble collagens have shown that there are mainly α-chains in collagens soluble in neutral salt solution, whereas the ratios of α- and β-components in collagens soluble in acid buffer solution are equal. This is in agreement with the recent view that the neutral salt-soluble collagen represents a recently synthesized collagen and that the number of cross-links in collagen increases with age as found, *e.g.*, by starch gel electrophoresis (Heikkinen and Kulonen 1964). Bakerman (1964), however, was not able to demonstrate any differences in the ratio of α- and β-components in soluble skin collagens from human beings of different ages.

Since the discovery of the dissimilarity of the α-chains, CM-cellulose chromatography or/and gel electrophoresis have been used to study the compositions of collagen chains in the following vertebrates: man (Bornstein and Piez 1964), cow (Schleyer 1962, Heidrich *et al.* 1965, Veis and Anesey 1965), rat (Piez *et al.* 1961, Näntö *et al.* 1963), lathyritic chick (Tanzer, Monroe and Gross 1966), cod (Piez 1965), carp (Piez *et al.* 1963) and spiny dogfish (Piez *et al.* 1963, Lewis *et al.* 1964).

The existence of α-, β- and γ-components has been confirmed in all these species. In determinations of the approximate component distributions in acid-soluble collagens of rat skin and carp ichthyocol, Piez *et al.* (1963) found that the ratio of $\alpha 1$ to $\alpha 2$ varies from 2.0 to 2.3 whereas the acid-soluble collagen of young spiny dogfish skin contains about ten times more $\alpha 1$ than $\alpha 2$. The behaviour of dogfish collagen on CM-cellulose column chromatography was also quite different and a high concentration of salt was required to elute the components from the column. The molecular structure $(\alpha 1)_2\alpha 2$ is generally accepted for tropocollagen regardless of species. Cooper and Davidson (1965), however, have proposed the structure $\alpha 1(\alpha 2)_2$ for the collagen of premature calves, whereas the structure of collagen in a full-term calf is $(\alpha 1)_2\alpha 2$.

Preliminary reports on the dissimilarity of the component compositions of guinea pig, pike, rayfish, hagfish and lamprey collagens have been published (Pikkarainen and Kulonen 1964, 1965, 1967, Pikkarainen, Rantanen and Kulonen 1966).

Stability of collagen structure

Stability of the helix. Investigations on the stability of the secondary structure of the native collagen molecule which is maintained mainly by hydrogen bonds usually deal with processes associated with the collagen-gelatin transitions.

The collagen-gelatin transition is a melting process in which collagen changes into a disorganized random coil (Flory and Garrett 1958). This transition may be detected at various levels of structural organization, *e.g.*, by measuring the thermal shrinkage

Table IV.

Denaturation temperatures of vertebrate collagens in the solid (T_S) and dissolved (T_D) states.

The T_S values are thermal shrinkage temperatures determined for skins in water or physiological saline. The T_D values were determined either by viscosimetry or polarimetry and refer to acid-buffered tropocollagen solutions.

	T_S	T_D	References
Mammals			
Man	60—67	36—39	1—6
Ruminants	59—70	34—39	3, 7—18
Pig	62	—	19
Horse	62—64	—	9
Dugong	69	—	20
Badger	58—60	—	9
Fin whale	62—64	—	9
Rat	59	37—38	3, 21—22
Rabbit	60—62	—	9
Reptiles			
Crocodile	59*	—	19
Python	57—59*	—	19
Lizard	60—62	—	9
Amphibians			
Toad	54*	—	19
Bony fishes			
Lungfish	63*	—	23
Flatfish	38—43	—	9
Perch	48—57	30—31	2—3, 9, 21, 24
Cod	37—45	12—17 (29)	3, 7—9, 11—12, 21, 25—27 (13)
Eel	55—57	26	9, 21
Carp	49—58	29—32	3, 9, 13, 15, 28
Pike	45—57	27	3, 9, 19, 12
Herring	—	18	21
Sturgeon**	50*	—	23
Cartilaginous fishes			
Shark	53*	29	23, 25
Dogfish	35—41	16	3, 29—31

*In toluene. **Swim bladder.

1 Hall and Reed (1957); *2* Rigby (1962); *3* Rigby (1967); *4* Bakerman (1961a); *5* Bakerman (1961b); *6* Bornstein and Piez (1964); *7* Gustavson (1942); *8* Gustavson

which is a macroscopic manifestation of collagen transformation or by the determination of the denaturation temperature of dissolved collagen.

A comparison of the thermal stabilities of vertebrate collagens is presented in Table IV. The values are relatively constant (shrinkage temperature in the range of 60°—70° and denaturation temperature 36°—39°) except for fish collagens.

Gustavson (1954b, 1955) studied thermal shrinkage very carefully, especially that of skins of various fishes. He suggested that the hydrothermal stability depends on the interchain hydrogen bonding of hydroxyl groups of hydroxyproline. Burge and Hynes (1959b) proposed a fundamental relationship between the stability and the total imino acid content rather than the hydroxyproline content. This was confirmed by Piez and Gross (1960), Maser and Rice (1962, 1963b) and Josse and Harrington (1964). According to Harrington (1964), the pyrrolidine residues stabilize the triple helical structure by decreasing the total configurational entropy change in the helix-coil transition. In addition to the stabilizing effect of hydrogen bonds between amide and carbonyl groups of adjacent strands, water molecule chains may be of importance as they may form interchain hydrogen bonds (Berendsen and Migchelsen 1966). The effects of van der Waals forces as well as of cross-links between the individual strands seem to be unimportant for stability (Harrington and McBride 1966).

Solubility of collagen. The solubility of collagen can also be considered a measure of stability depending on the quaternary structure (cross-linking) of the collagen. The tropocollagen molecules are in fibrous form in the tissue. The fibres are bound by intermolecular cross-links which are responsible for the relative insolubility of collagen in aqueous and organic solvents (Hörmann 1962, Harding 1965). Collagen can, however, be separated into various artificial fractions by employing different solvents.

The solubility of collagen in dilute acids has been known almost a century. More attention was paid to soluble collagens after Russian workers showed that a part of skin collagen dissolves in an acid buffer solution. The results of these studies on this "procollagen" from several sources have been summarized by Orekhovitch and Shpikiter (1958b).

A portion of this "procollagen" was extracted from the tissues also by alkaline phosphate and neutral salt solutions. The neutral salt-soluble collagen is the precursor of mature collagen. The biological significance of acid-soluble "procollagen" is as yet unknown. It has been considered not only a precursor of collagen but also a degradation product. (In the present study the acid-soluble or acid citrate buffer-soluble collagen refers to collagen extracted from the skin after the removal of the neutral salt-soluble collagen.)

In comparisons of soluble and insoluble collagens, very minute, perhaps insignificant, differences have been found, *e.g.,* in amino acid composition (Polatnick, La Tessa and Katzin 1957, Jackson *et al.* 1958, Orekhovitch *et al.* 1960), molecular weight and molecular dimensions (Hannig and Engel 1961, Kawai *et al.* 1966).

(1950); *9* Gustavson (1953); *10* Gustavson (1954a); *11* Ward (1958); *12* Esipova (1957); *13* Doty and Nishihara (1958); *14* Crosby and Stainsby (1962); *15* v. Hippel and Wong (1963a); *16* v. Hippel and Wong (1963b); *17* Gross and Nagai (1965); *18* Fujimori (1966); *19* Leach (1957); *20* Joseph *et al.* (1964); *21* Burge and Hynes (1959a); *22* Piez and Carrillo (1964); *23* Eastoe (1957); *24* Burge and Hynes (1959b); *25* Gustavson (1955); *26* Young and Lorimer (1960); *27* Young and Lorimer (1961); *28* Veis and Drake (1963); *29* Takahashi and Tanaka (1953); *30* Lewis and Piez (1961); *31* Lewis and Piez (1964).

The high solubility of fish skin collagen on heating has been known for a long time (Gustavson 1942). Table V presents data on the solubilities of skin collagens of full-grown vertebrates. These show that the amount of native soluble collagen in mammalian skin is remarkably low compared to its amount in fish skin.

Table V.

Contents and solubilities of native collagens of skins of full-grown vertebrates.

	Percent collagen in		Percent collagen soluble in		References
	wet skin	dry skin	neutral salt solutions	acid buffer solutions	
Man	12—16	40—95	0.11—0.26*	0.02*—3.2	1—6
Cow	—	65—91	0.03	0.5—5	7—10
Guinea pig	—	33—68	7—8	1	13—15
Rat	15—26	40—75	0.05**—7.6	0.88**—6.7	16—27
Mouse	1—7	43—58	0.2	2.3	27—29
Rabbit	15	37	2.15—4.3	5.33	15, 30
Cod	—	74	—	62	31

* Soluble collagen as a percentage of the dry weight of the skin.
** Soluble collagen as a percentage of the wet weight of the skin.

1 Bakerman (1964); *2* Korting, Holzmann and Kühn (1964); *3* Harris and Sjoerdsma (1966); *4* Sobel *et al.* (1958); *5* Clausen (1962); *6* Majewski, Leja and Majewska (1964); *7* Grassmann (1960); *8* Veis, Anesey and Cohen (1960); *9* Crosby and Stainsby (1962); *10* Smits *et al.* (1957); *11* Bowes, Elliott and Moss (1958); *12* Verzar (1960); *13* Gross (1958); *14* Orekhovitch and Shpikiter (1958b); *15* Sakata (1960); *16* Wirtschafter and Bentley (1962); *17* Holzmann *et al.* (1964); *18* Kühn *et al.* (1964a); *19* Kühn *et al.* (1964b); *20* Nimni and Bavetta (1964); *21* Verzar (1964); *22* Mills and Bavetta (1966); *23* Kao, Hilker and McGavack (1960); *24* McGavack and Kao (1960); *25* Murray, Watts and Ring (1961); *26* Cadavid, Denduchis and Mancini (1963); *27* Dickerson and John (1964); *28* Hamer and Marchant (1957); *29* Harkness, Harkness and James (1958); *30* Nimni, DeGuia and Bavetta (1966); *31* Young and Lorimer (1960).

The quantity of soluble collagen varies with the nutritional state of the animal (Gross 1958b) and with the site from which the skin piece is taken (Verzar 1964). The amount of acid-soluble collagen depends greatly on the particle size of the disintegrated tissue that is extracted. Riess (1964) and Küntzel (1964) have demonstrated that up to 95 % of bull hide collagen dissolves after intensive homogenization.

Insoluble collagen that remains after thorough extraction of soluble collagens may be dissolved as gelatin. Pikkarainen *et al.* (1964) were able to demonstrate that the starch gel electrophoretic pattern of insoluble collagen of guinea pig skin heated to 40° reveals the existence of α- and β-components in addition to larger aggregates. High molecular weight aggregates have also been found in extracts obtained on heating bull hide corium in water at 60° (Veis *et al.* 1960).

THE PURPOSE OF THE PRESENT INVESTIGATION

The purpose of the present investigation was to obtain knowledge of the evolution of collagen. Comparative chemical investigations of skin collagens of several vertebrates at different levels of protein structure were carried out to find answers to the following questions:

1. Do the *amino acid compositions* of collagens correlate with animal evolution? What are essential features of collagen structure?

2. Has the *helical structure* remained constant during evolution as judged by the optical rotatory properties of tropocollagen solutions? How much does the *thermal stability* of the helical structure of collagens vary as measured by optical rotation and thermal contraction? How does the stability correlate with chemical structure?

3. Are the *components* of tropocollagen influenced by evolution? Is an ancestral polypeptide chain detectable?

4. Are the *solubilities* of skin collagens related to the evolutionary stage?

MATERIAL AND METHODS

SAMPLES OF SKINS

The specimens were collected alive when possible. Otherwise, only fishes that had been dead at most one hour and had been stored meanwhile on ice were used. The samples included the following materials:

Cattle (*Bos taurus* L.), forehead and neck skin of a cow (age unknown) and a calf (age one week);

Guinea pig (*Cavia porcellus* (L.)), skins of 8 full-grown (weighing 520—840 g, mean 650 g) and 15 growing (weight under 500 g) guinea pigs;

Chick (*Gallus domesticus* L., White Rock), skins of two broilers (weighing about 1.5 kg);

Ringed snake (*Natrix natrix* (L.)), skins of 5 snakes (lengths over 50 cm);

Toad (*Bufo bufo* (L.)) and *Frog* (*Rana temporaria* L.), skins of 8 toads and skins of 18 frogs (weighing 15—34 g, mean 23 g);

Flounder (*Pleuronectes flesus* L.)[1], skins of 3 flounders (weighing over 500 g);

Burbot (*Lota vulgaris* Jenyns)[1], skin of one burbot (weight 1.7 kg);

Pike (*Esox lucius* L.)[1], skins of 2 pikes (weighing 1—2 kg);

Smooth dogfish (*Mustelus mustelus* (L.))[2], skins of 4 fishes (lengths 40—50 cm). The skins were preserved about one week in 15 % ethanol at 4° before final treatment (Pikkarainen and Kulonen 1966);

Rayfish (*Raja* sp.)[3], abdominal skins of 6 rayfishes (lengths over 40 cm, widths over 40 cm);

Hagfish (*Myxine glutinosa* L.)[3], skins of 30 hagfishes (lengths 25—30 cm);

Lamprey (*Petromyzon fluviatilis* L.)[4], skins of 35 lampreys (lengths over 35 cm).

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[1] From the Turku archipelago in Finland.

[2] From the Mediterranean at the Marine Biological Station of Parma University at St. Margherita lig. (courtesy of Fisherman Andrea Ghiardello).

[3] From the Skagerrak at Kristineberg Zoological Station on the west coast of Sweden (courtesy of Dr. Bertil Swedmark).

[4] From the Pori archipelago in Finland (courtesy of Fisherman Erkki Lundgren).

Treatment of skins. The animals were skinned immediately after slaughter, the hagfishes and lampreys while still alive. The hair, scales and subcutaneous fat were removed carefully. The skins were rinsed with water, cut into pieces and homogenized. The frozen mammalian skins were ground in an ice-cold meat-grinder before homogenization. The material was homogenized in a rotating blade disintegrator (Edmund Bühler, Tübingen, West Germany, 50,000 rev./min).

Dry matter, ash content and hydroxyproline determinations were carried out on the rinsed material.

FRACTIONATION OF COLLAGEN BY SOLVENT EXTRACTION

The isolation and purification of the various collagen fractions were performed at 4° unless otherwise stated. All extractions were carried out overnight with agitation in a swirling shaker (C. Desaga, GmbH, Heidelberg, West Germany, 80—140 strokes per minute). In the centrifugations either an MSE "High-Speed 17" or an MSE "Superspeed 50 S" refrigerated centrifuge (Measuring & Scientific Equipment Ltd., London, England) was used.

Isolation and purification of neutral salt-soluble collagen

The isolation of NSC from guinea pig and lamprey skins was carried out by repeated extraction with 0.45 M sodium chloride solution as described in Fig. 1. The neutral salt-soluble collagen in combined extracts was purified according to Gross (1958a) as rapidly as possible. The total recoveries were 74.2 % and 79.9 %, respectively, for guinea pig and lamprey collagens. As a criterion of the purity the ratio of hydroxyproline nitrogen to total nitrogen was determined. The ratios for NSC of guinea pig and lamprey were 0.082 and 0.071.

Isolation and purification of citrate-soluble collagen

Citrate-soluble collagen was isolated by repeated extraction with 0.15 M citrate buffer of pH 3.7 of the tissue mass remaining after the extractions with sodium chloride solutions, as described in Fig. 1. The combined citrate extracts were purified according to the scheme in Fig. 2.

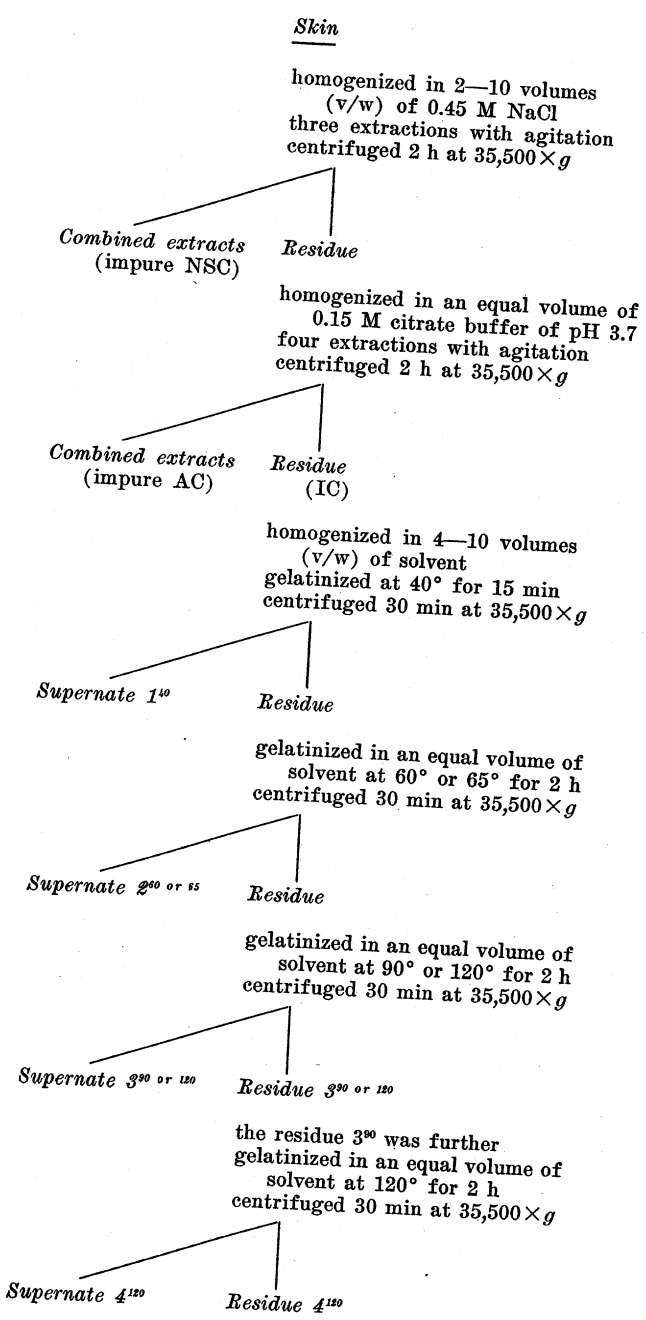


Fig. 1. Fractionation scheme for skin collagen.

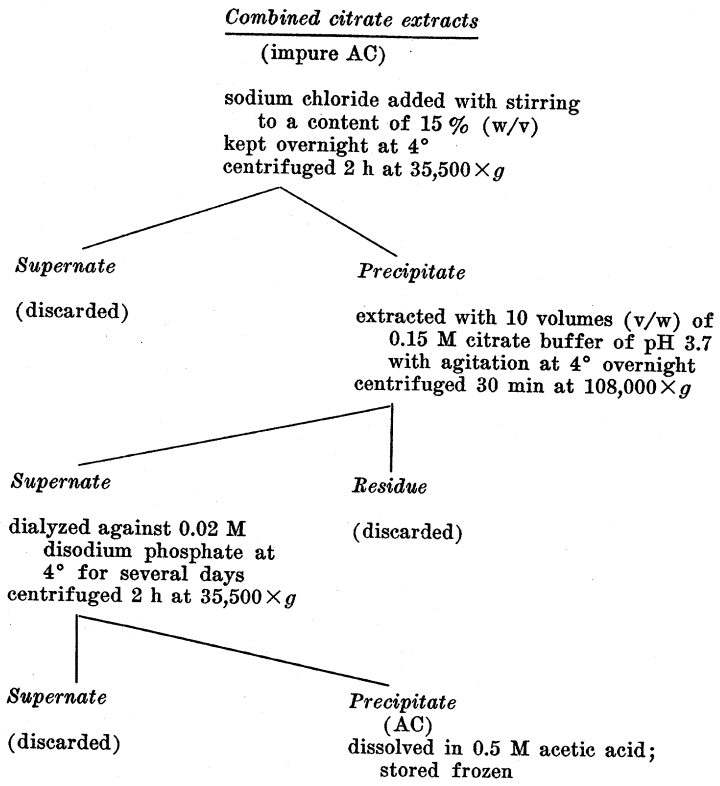


Fig. 2. Purification scheme for acid-soluble collagen.

The recoveries of citrate-soluble collagen after precipitation with sodium chloride and dialysis against disodium phosphate solution were 97.7 % and 96.9 %. The ratio of hydroxyproline nitrogen to total nitrogen varied from 0.063 to 0.112.

Isolation of insoluble collagen

After the citrate extractions the tissue mass was suspended in a gelatinization medium, agitated at 4° and centrifuged. This was repeated until the extract no longer contained protein (biuret reaction). The residue was gelatinized as shown in Fig. 1.

When the gelatinization was carried out in a 0.01 M sodium acetate buffer solution of pH 4.8, the ratio of hydroxyproline nitrogen to total nitrogen varied from 0.052 to 0.089 in supernates 1^{40} and from 0.046 to 0.098 in supernates $2^{60 \text{ or } 65}$.

GENERAL ANALYTICAL METHODS

Dry matter and ash

A known weight of wet skin (0.1—5.0 g) was dried in a quartz crucible to constant weight (usually two days) at 95°. Dry matter determinations were carried out at least in triplicate. The coefficient of variation (100× standard deviation/arithmetic mean) of the method was ± 5.1 %.

The dry matter was combusted at 530° in a muffle furnace (W. C. Heraeus, GmbH, Hanau, West Germany, Type MB 170) overnight. Weighing was always done after a constant period of time in a desiccator. The coefficient of variation in the determination of the ash content was ± 10.8 %.

Hydroxyproline

The samples were hydrolysed in 5.7 N hydrochloric acid in tubes (culture tubes with screw caps and PTFE packings, Kimax®) at 130° for 3 hours. The hydrochloric acid was volatilized on a boiling water-bath. The dry residue was dissolved in distilled water immediately before the colour reaction was carried out by the method of Stegemann (1958) as modified by Woessner (1961).

A standard gelatin (acid-processed pig-skin gelatin No. 149 (see Biochem. J. 1961, *79*, 652—656), a gift from Dr. J. E. Eastoe) was included in every series. The coefficient of variation of the method was ± 2.2 %.

Nitrogen

The combustions were carried out by the method of Stegemann (personal communication to Dr. V. Näntö). Half a millilitre of 8 N sulphuric acid was added to a sample containing 5—30 μg of nitrogen and the test tube was transferred to a hole in a aluminium block. The temperature of the block was raised to 300° on an electric plate and the samples were combusted until no organic material remained.

Then 3.0 ml of water was added to each tube followed by 3.0 ml of Nessler's reagent (made 0.015 M in potassium cyanide to prevent precipitation as proposed by Minari and Zilversmit (1963)). The absorbance was measured at 420 nm against a reagent blank after 30 min.

A series of ammonium sulphate standards was included in every digestion series. The coefficient of variation of the method was ± 1.7 %.

α-Amino nitrogen

The α-amino nitrogen contents were determined according to Szentirmai *et al.* (1962). Six glutamic acid standards of varying concentration were included in every series. The coefficient of variation of the method was ± 6.9 %.

Protein

The biuret method was a modification of that described by Lowry *et al.* (1951).

A known volume, 0.030—2.00 ml, of solution containing 0.005—0.2 mg of protein was pipetted into a test tube. After the addition of 3.0 ml of freshly prepared alkaline copper reagent (50 volumes of 6 % sodium carbonate in 0.25 N sodium hydroxide solution and one volume of 0.5 % copper sulphate in 1 % sodium or potassium tartrate solution), the contents were mixed and left to stand for 10 min. Diluted Folin's reagent (0.6 ml) was added and the mixture shaken immediately. The absorbance at 750 nm was measured against the reagent blank after 30 minutes.

Standard curves were made for the various sample volumes using standard gelatin (see the determination of hydroxyproline). The coefficient of variation of the method was ± 3.1 %.

CONTENT OF COLLAGEN

The collagen content was calculated either from the content of hydroxyproline (by multiplying the hydroxyproline content by 7.3) or from the content of total nitrogen (by multiplying the nitrogen content by 5.55) or determined by the biuret method.

DETERMINATION OF AMINO ACID COMPOSITION

Hydrolysis. Protein (1—3 mg) was hydrolysed in 2—4 ml of 5.7 N hydrochloric acid under nitrogen at 110° for 20 hours. After the hydrochloric acid had been volatilized on a boiling water-bath, the dry residue was dissolved in 0.2 N citrate buffer of pH 2.2 and stored frozen. The α-amino nitrogen content of the hydrolysate was determined.

Description of the equipment and method. Amino acid analyses were carried out by ion exchange chromatography with an automatic amino acid analyser built according to Spackman, Stein and Moore (1958).

Ground Amberlite resin CG-120, Type III, 400 mesh, was fractionated by the hydraulic method of Hamilton (1958). Fractions of particle sizes 68 ± 18 μ and 47 ± 11 μ were used to resolve neutral and basic amino acids, respectively.

The buffer solutions were prepared exactly as proposed by Moore, Spackman and Stein (1958) except that the pH of the first buffer solution used to elute the 150-cm column was 3.05 ± 0.02 instead of 3.25.

The solvents were pumped through the 150-cm column with an Accu-Flo® pump (Beckman Instruments Inc., Spinco Division, Palo Alto, Calif., U.S.A.) and through the 50-cm column in the early stages of the investigation with a MiniFlow precision micropump (LKB Produkter AB, Stockholm, Sweden, Type 4501) at a flow rate of 22.4 ml/h. The ninhydrin reagent was also pumped with a MiniFlow pump at a rate of 11.2 ml/h. Later the MiniFlow pumps were replaced by Accu-Flo® pumps and the flow rates were increased to 40 ml/h and 20 ml/h, respectively.

The mixture of column effluent and ninhydrin reagent was led through PTFE tubing, bore initially 0.5 mm and later 0.8 mm when the flow rate was increased, through a boiling water-bath where the mixture remained for at least 15 minutes. The colorimeter was constructed precisely according to the instructions of Spackman *et al.* (1958). Signals were registered with a multichannel recorder (Philips Type PR 3210 A/00), using a chart speed of 40 mm/h, with a 1-mm interval between two successive printings on the same channel. A special kind of logarithmic chart paper manufactured by A. Ahlström Oy, Euran Paperi, Kauttua, Finland, was used.

The separation of acidic and neutral amino acids was carried out in a 150×0.9 cm resin column at $50 \pm 1°$. The initial eluting solution was a 0.20 N citrate buffer of pH 3.05 ± 0.02. A change to 0.20 N citrate buffer of pH 4.25 ± 0.02 was performed automatically so that cystine and valine were eluted by the second buffer. A 50×0.9 cm resin column was used in the analysis of basic amino acids. The eluting buffer was a 0.38 N citrate buffer of pH 4.26 ± 0.02. When lysine had emerged from the column, the temperature was raised automatically from 30° to 50°.

Standardization and calculation of results. Norleucine (L. Light & Co., Ltd., Poyle, Colnbrook, Bucks., England) and β-2-thienylalanine (Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.) were included as internal standards in every chromatographic run through the 150-cm column and β-2-thienylalanine and 2-amino-3-guanidopropionic acid (Cal-

biochem, Los Angeles, Calif., U.S.A.) in every run through the 50-cm column (Siegel and Roach 1961, Walsh and Brown 1962).

For quantitation of the chromatographic peaks, a standard mixture of amino acids (Amino Acid Calibration Mixture Type I for Amino Acid Analyzer, Lots CM 115 and CM 116, Beckman Instruments Inc., Spinco Division, Palo Alto, Calif., U.S.A.) was repeatedly chromatographed. The following standard amino acids, which were found to be pure by two-dimensional paper chromatography, were added to this mixture: cysteic acid (L. Light & Co., Ltd., Poyle, Colnbrook, Bucks., England), methionine sulphoxide, hydroxyproline (Fluka AG, Buchs SG, Switzerland), delta-hydroxylysine (K & K Laboratories Inc., Plainview, New York, U.S.A.) and ornithine (S.A. F. Hoffmann-La Roche & Co., AG., Basle, Switzerland).

The peak areas were calculated by multiplying the height (absorbance) of each peak by its width at half height. A colour constant was obtained by dividing the number of micromoles of an amino acid by the respective peak area. Relative colour constants were calculated for every amino acid by taking the constant for norleucine to be unity. The amount of each amino acid in micromoles in the sample was obtained from the formulas:

150-cm column	*50-cm column*
$X = q_x \cdot a_x / a_n$	$X = q_x \cdot a_x \cdot y / a_\beta \cdot q_\beta$

X = number of micromoles of amino acid x
q_x = relative colour constant of amino acid x (norleucine = 1.00)
q_β = relative colour constant of β-2-thienylalanine (norleucine = 1.00)
a_x = measured peak area of amino acid x
a_n = measured peak area of 1.00 μmole of norleucine
a_β = measured peak area of 1.00 μmole of β-2-thienylanine
y = the ratio of the α-amino nitrogen content of the sample added to the 150-cm column to the α-amino nitrogen content of the sample added to the 50-cm column

The amino acid composition was expressed by giving the number of residues of the individual amino acids per 1000 residues.

The mean coefficients of variation of the relative colour constants were $\pm$ 3.4 % (range 0.8—6.0 %) at 570 nm and $\pm$ 14.2 % (range 12.0—16.3 %) at 440 nm when using the 150-cm column, and $\pm$ 6.1 % (range 4.9—8.4 %) when using the 50-cm column.

DETERMINATION OF OPTICAL ROTATORY DISPERSION

Samples. Citrate-soluble collagens in 0.5 M acetic acid were dialyzed at 4° against a 0.15 M citrate buffer solution of pH 3.7 and centrifuged at 200,000×g until clear. The samples were then diluted to a collagen concentration of about 0.1 % (this concentration was later accurately calculated from the nitrogen content of the sample actually used in polarimetry).

Equipment and measurement. The Perkin-Elmer Type 141 polarimeter (Perkin-Elmer Co., GmbH, Überlingen, Bodensee, West Germany) was equipped with both mercury and sodium lamps and filters 313, 365, 436, 546, 578 and 589 nm. A jacketed 1.3-ml glass tube (length 99.75 mm and inner diameter 4 mm) was constructed in our workshop.

The optical rotations of the native collagen solutions were measured at 365, 436, 546 and 578 nm at 8° (at 4° in the case of rayfish collagen). Before similar determinations of the optical rotations of denatured collagen solutions, the collagens were denatured at 40° for 30 minutes.

Calculation of results. The optical rotations of both native and denatured collagen solutions were studied at a series of wavelengths to determine whether they can be represented by the *Drude* equation

$$[\alpha]_\lambda = A/(\lambda^2 - \lambda_c^2)$$

$[\alpha]_\lambda$ = specific rotation at wavelength λ
λ = wavelength of incident radiation
λ_c = constant characteristic of the material (wavelength of absorption maximum)
A = a constant characteristic of the material that includes the refractive index (dependent on the helix content of proteins).

The value of λ_c is determined from the slope of the plot of $\lambda^2[\alpha]_\lambda$ against $[\alpha]_\lambda$ (Yang and Doty 1957).

The helix content was estimated by substituting the optical rotatory dispersions of native and denatured collagens in the *Moffitt-Yang* equation

$$[\alpha]_\lambda = Ka_0\lambda_0^2/(\lambda^2 - \lambda_0^2) + Kb_0\lambda_0^4/(\lambda^2 - \lambda_0^2)^2$$

b_0, λ_0 = constants that are principally functions of the helical backbone, not dependent on side chains or environment, *i.e.*, solvent, temperature and pH
a_0 = constant representing intrinsic residue rotations, amino acid com-

position, and rotations due to interactions within the helix, and dependent upon the environment

K = constant that is a function of the mean residue weight of collagen and of the refractive index of the solvent.

The equation was solved graphically by determining the wavelength λ_0 which gave a linear plot when $[\alpha]_\lambda(\lambda^2—\lambda_0^2)$ was plotted against $(\lambda^2—\lambda_0^2)^{-1}$.

The mean values of λ_0 were found to be 251 nm and 254 nm for the studied native and denatured collagens, respectively (Table VIII). The value 250 nm of λ_0 was used in the calculations because a variation of ± 5 nm is permitted by Moffitt and Yang in the choice of λ_0 and since it is essential to use the same value of λ_0 for both native and denatured macromolecules when determining the helix content (Urnes and Doty 1961). The constants Kb_0 and Ka_0 were obtained by dividing the graphically determined slope of the plot by λ_0^4 and the intercept by λ_0^2, respectively (Fasman 1963).

For the comparison of the helix contents of the various collagens a quantity related only to the helix content was formed as follows. The obtained values of constants (Kb_0^D and Ka_0^D) for denatured collagen were subtracted from those (Kb_0^H and Ka_0^H) for native collagen. This gives two terms both dependent on the helix content, $Kb_0^H—Kb_0^D$ (written Kb_0^{H-D}) and $Ka_0^H—Ka_0^D$ (written Ka_0^{H-D}), which could be combined into one (b_0^{H-D}/a_0^{H-D}) by dividing the former by the latter. This procedure is based on the assumption that the value of the constant K is the same for both native and denatured collagens. Actually the value of K varies with temperature since it includes the refractive index which is known to be temperature dependent. When, however, the optical rotatory dispersions are determined under comparable conditions, the error due to the variation of K is the same in all measurements and can be neglected.

DETERMINATION OF THE DENATURATION TEMPERATURE OF DISSOLVED COLLAGEN

Samples. The denaturation temperatures of collagen solutions were determined on the same samples as were used for the determination of the optical rotations of native collagens.

Equipment and procedure. The polarimeter and the jacketed tube were those described on p. 26. The jacket was connected to a water-bath

and the circulation both in the bath and through the tube was maintained by two pumps (H. Heidolph, Schwabach, West Germany, Type P 50, 270 rev./min). A coil through which cold tap water passed was immersed in the bath to minimize temperature fluctuations.

The optical rotation of a collagen solution was first measured at 365 nm against a 0.15 M citrate buffer of pH 3.7 at 8° (at 4° in the case of rayfish collagen). The temperature was then raised two degrees in two minutes, and five minutes after the new temperature was reached, the temperature was measured again. The temperature was raised again (two degrees in two minutes) exactly after ten minutes had elapsed from the beginning of the preceding rise, and the rotation was measured seven minutes after the temperature had been raised. This procedure was repeated until the rotation no longer changed.

Calculations. The specific rotations were calculated and the denaturation curve was constructed by plotting $([\alpha]^{t}_{365} - [\alpha]^{D}_{365})/([\alpha]^{H}_{365} - [\alpha]^{D}_{365})$ as a function of temperature.

$[\alpha]^{t}_{365}$ = specific rotation at 365 nm and temperature t

$[\alpha]^{D}_{365}$ = specific rotation of completely denatured collagen at 365 nm

$[\alpha]^{H}_{365}$ = specific rotation of native collagen at 365 nm.

Because of the dependence of the optical rotation on the secondary structure of collagen, this ratio gives the helix content at a given temperature. The denaturation temperature (T_D) of a collagen solution was defined as the temperature where a 50 % change in optical rotation had occurred.

DETERMINATION OF THE SHRINKAGE TEMPERATURE OF SKIN

Samples. The hair, feathers or scales and the subcutaneous fat were removed from the skins which were then rinsed in running tap water and stored frozen until analysed. Before analysis, the thawed specimens were kept in distilled water at 4°, which was replaced repeatedly, for at least 24 hours. The skin of the smooth dogfish was an exception, for it was preserved in 15 % aqueous ethanol at 4° about one week before rinsing (Pikkarainen and Kulonen 1966).

Equipment and measurements. A piece of skin, 1 × 5 cm, with punched holes in each end (F, Fig. 3) was hung with the aid of two hooks from a rubber stopper (I). A 60—500 mg stainless steel weight (G) was attached

FRACTIONATION OF COLLAGEN COMPONENTS BY STARCH GEL ELECTROPHORESIS

Samples. The samples of collagen were either dialyzed against or dissolved in a sodium acetate buffer of pH 4.7 (I = 0.017) to give an about 0.5 % solution. Before fractionation the sample solution was denatured by keeping it at least 15 minutes at 40°.

Method. The starch gel electrophoresis of collagen has been described in detail earlier (Näntö *et al.* 1963, 1965). Eleven grams of starch (Connaught Medical Laboratories, Ltd., Toronto, Canada) suspended in 75 ml of a sodium acetate buffer solution was heated in a flask over a flame with continuous stirring. When the formed gel began to boil, it was deaerated with a water pump half a minute. The gel was poured into a trough, covered with a glass plate and allowed to cool at least 1.5 hours. It was rewarmed to 38° before application of the denatured collagen sample solution imbibed in a piece of Whatman 3 MM filter paper. The moist filter paper was inserted in a perpendicular slot in the anode end of the gel and the gel sheet was covered with a thin film of plastic before applying the current.

The combined volume of the buffer compartments was 3.5 l. The voltage applied to the electrodes was 125 V, and the duration of electrophoresis was usually six hours. The electrophoresis was carried out in a room held at 38°.

The stained and washed gels were photographed on Adox KB-14 or Agfa Agepan films using a dark red filter (Johannes Weber KG, Wiesbaden, West Germany) for better contrast. The absolute and relative migrations were calculated for the various components. In the calculation of relative migration the distance travelled by the fastest moving band in each gel was taken as 100.

Standardization. An attempt was made to keep the voltage constant during the run by manual adjustment. In order to get comparable electrophoretic migration data for various collagens, purified citrate-soluble collagen of guinea pig skin was used as an internal standard in every electrophoretic run in each gel sheet.

When the effect of ionic strength or pH on the electrophoretic pattern was studied, all the respective runs were carried out simultaneously. The conductances of the buffers used were also measured.

FRACTIONATION OF COLLAGEN COMPONENTS BY CARBOXYMETHYLCELLULOSE COLUMN CHROMATOGRAPHY

Samples. The samples of citrate-soluble collagens were dialyzed against 0.01 M sodium acetate buffer of pH 4.7 at 4°. Before chromatography the dialyzed solutions were denatured by keeping them at least 15 minutes at 40°.

Equipment and procedure. A jacketed chromatographic tube (inside diameter 20 mm) held at 38° was packed under nitrogen pressure to a column height of 30 cm with CM-cellulose (Whatman CM 70 or Whatman Chromedia grade CM 11) that had been preswollen in the starting buffer at least one night. The packed column was then equilibrated with the starting buffer.

A linear gradient (Piez *et al.* 1963) between the starting and limiting buffers was obtained with a peristaltic pump (C. Desaga, Heidelberg, West Germany) as described by Davis, Santen and Agranoff (1965). The limiting buffer was prepared by adding sodium chloride to the starting buffer, a sodium acetate buffer of pH 4.8 with $I = 0.03$, 0.04 or 0.06, until the sodium chloride concentration was 0.10 molar ($I = 0.13$, 0.14 or 0.16). Sometimes the column was eluted further with 1.7 M sodium chloride solution applied directly onto the bed after the gradient. The effluent was collected in 2- or 4-ml fractions in a collector (Dr. Hans Hösli, Bischofszell, Switzerland, Type TVZ-260) at a constant flow rate of 60 ml/h. All connections to and from the column were small-bore (1.0—1.5 mm) polythene tubing. The buffers were deaerated by heating and the liquid in the mixing vessel was covered with liquid paraffin.

Protein in the effluent was detected either with the biuret reaction or, before the collection of the fractions, by recording the transmittance at 230 nm with a UV—Vis 139 Hitachi—Perkin—Elmer spectrophotometer equipped with a photomultiplier, a flow cell (light path 5 mm) and a Hitachi—Perkin—Elmer Type 159 recorder.

Combined effluent fractions were freeze-dried to reduce the volume and desalted on Sephadex G-25 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) columns at 38° using pyridine-acetate buffer of pH 4.7 (0.5 % pyridine in 0.45 % acetic acid) for elution. The desalted solutions were freeze-dried again and the residue was dissolved, with slight warming when necessary, in either 0.5 M acetic acid or the sodium acetate buffer of pH 4.7 ($I = 0.017$) used in the starch gel electrophoresis. The fractions were stored at —20°.

RESULTS

AMINO ACID COMPOSITIONS

The amino acid compositions of the citrate-soluble collagens of several vertebrates are presented in Tables VI and VII. The results confirm the known constancy of the content of nonpolar amino acids and the sum of the contents of acidic and hydroxy amino acids as well as the absence of cystine (Tables I and II). The content of glycine is about one third of the total amino acid content in different collagens. Not only the glycine content, but also the contents of alanine, glutamic acid and aspartic acid vary only slightly.

Pig and chick collagens have high contents of both imino acids, but low contents of serine and threonine compared to collagens of fishes and cyclostomes. The methionine contents are also lower in the pig and chick collagens than in the collagens of fishes, except flounder.

The collagens of the cartilaginous fishes, dogfish and rayfish, have higher contents of isoleucine and arginine (and hence basic amino acids) than the collagens of all the other species examined in the present study and contain more glutamic acid, valine and leucine than flounder or cyclostomes. The amino acid compositions of dogfish and rayfish collagens seem to differ only in the serine, threonine and imino acid contents that are believed to be responsible for the stability of collagen.

The collagens of cyclostomes, hagfish and lamprey, have lower contents of leucine than the other collagens studied. Lamprey collagen resembles the collagens of higher vertebrates more than hagfish collagen in having a higher content of imino acids and less serine and threonine.

An evolutionary trend in the amino acid composition of collagen can be observed in the contents of imino acids, threonine, serine, methionine and histidine. Flounder collagen is not typical as in the contents of threonine, methionine and histidine it resembles more the collagens of higher vertebrates than the collagens of fishes. The composition of lamprey collagen also differs from the expected in the contents of various amino acids (see Discussion).

Table VI.

Amino acid compositions of citrate-soluble collagens.

The values are numbers of amino acid residues per 1000 residues and have not been corrected for losses during hydrolysis and chromatography. The numbers in parentheses are the numbers of determinations.

	Pig* (2)	Chick	Frog	Flounder (2)	Dogfish (2)	Rayfish	Hagfish (2)	Lamprey (2)
Hydroxyprolines (3- & 4-)	91	99	65	63	72	61	62	66
Aspartic acid	46	47	55	47	42	42	50	49
Threonine	18	19	22	20	25	30	27	23
Serine	35	27	56	65	46	71	71	58
Glutamic acid	72	73	62	58	72	72	59	67
Proline	132	113	102	103	99	84	92	107
Glycine	330	334	362	374	333	332	381	356
Alanine	111	127	122	120	119	97	116	121
Half cystine (and cysteic acid)	—	—	—	—	—	—	—	—
Valine	26	19	17	18	25	23	17	17
Methionine (and sulphoxides)	4	7	5	7	11	14	11	10
Isoleucine	10	12	11	7	18	19	10	8
Leucine	24	26	25	20	27	28	16	19
Tyrosine	3	<0.5	2	1	<0.5	5	2	1
Phenylalanine	13	10	11	7	10	14	8	9
Hydroxylysines	7	3	3	3	3	3	3	4
Ornithine	—	1	—	—	—	—	—	<0.5
Lysine	26	27	26	26	29	26	14	23
Histidine	4	3	3	4	7	10	7	6
Arginine	49	53	51	57	62	69	54	56
Acidic amino acids	118	120	117	105	114	114	109	116
Basic amino acids	86	87	83	90	101	108	78	89
Difference	32	33	34	15	13	6	31	27
Imino acids	223	212	167	166	171	145	154	173
Hydroxy amino acids	154	148	148	152	146	170	165	152
Nonpolar amino acids	650	648	655	656	642	611	651	647

* Pig-skin gelatin, see p. 22.

Table VII.

Variations in the amino acid contents of citrate-soluble collagens.

The values are based on the data in Table VI.

	Range	Δ	Mean	Δ/Mean
Hydroxyprolines (3- & 4-)	61— 99	38	80	0.48
Aspartic acid	42— 55	13	48.5	0.27
Threonine	18— 30	12	24	0.50
Serine	27— 71	44	49	0.90
Glutamic acid	58— 73	15	65.5	0.23
Proline	84—132	48	108	0.44
Glycine	330—381	51	355.5	0.14
Alanine	97—127	30	112	0.27
Half cystine (and cysteic acid)	—	—	—	—
Valine	17— 26	9	21.5	0.42
Methionine (and sulphoxides)	4— 14	10	9	1.11
Isoleucine	7— 19	12	13	0.92
Leucine	16— 28	12	22	0.55
Tyrosine	<0.5— 5	5	2.5	2.00
Phenylalanine	7— 14	7	10.5	0.67
Hydroxylysines	3— 7	4	5	0.80
Ornithine	0— 1	1	0.5	2.00
Lysine	14— 29	15	21.5	0.70
Histidine	3— 10	7	6.5	1.08
Arginine	49— 69	20	59	0.34
Acidic amino acids	105—120	15	112.5	0.13
Basic amino acids	78—108	30	93	0.32
Difference	6— 34			
Imino acids	145—223	78	184	0.42
Hydroxy amino acids	146—170	24	158	0.15
Nonpolar amino acids	611—656	45	633.5	0.07

OPTICAL ROTATORY DISPERSIONS

The optical rotations of several citrate-soluble collagens were determined at different wavelengths to evaluate the optical rotatory dispersions. Two equations were fitted to the optical data. The Drude equation describes the rotatory dispersion in regions distant from absorption bands, whereas the equation of Moffitt and Yang gives the helix content more reliably. The results of these calculations are presented in Table VIII.

Table VIII.

Optical rotatory dispersion values of citrate-soluble collagens.

The value $\lambda_o = 250$ nm was used in the calculation of the values of Kb_o and Ka_o.
The letters H and D refer to native and denatured collagens, respectively.

	Drude					Moffitt-Yang						
	λ_c^H	λ_c^D	$A^H \times 10^7$	$A^D \times 10^7$	$(A^H - A^D) \times 10^7$	λ_o^H	λ_o^D	Kb_o^H	Kb_o^D	Ka_o^H	Ka_o^D	b_o^{H-D}/a_o^{H-D}
Calf	198	205	—15.19	—5.96	— 9.23	257	296	647	276	—2392	—978	—0.26
Guinea pig, full-grown	188	200	—17.00	—5.55	—11.45	240	240	801	227	—2653	—870	—0.32
growing	200	214	—12.99	—4.71	— 8.28	248	262	519	150	—2029	—742	—0.29
Chick	201	210	—13.53	—4.88	— 8.65	245	257	540	166	—2122	—770	—0.28
Frog	212	204	—11.07	—4.32	— 6.75	272	225	366	169	—1746	—680	—0.18
Flounder	202	195	—13.49	—3.98	— 9.51	257	227	524	166	—2110	—618	—0.24
Burbot	202	230	—13.02	—3.26	— 9.76	249	276	519	84	—2046	—528	—0.29
Pike	203	206	—12.49	—4.23	— 8.26	248	251	491	150	—1963	—661	—0.26
Dogfish	202	214	—13.10	—4.04	— 9.06	245	265	517	133	—2054	—640	—0.27
Rayfish	200	203	—11.20	—3.44	— 7.76	242	250	470	126	—1766	—536	—0.28
Hagfish	201	215	—10.30	—3.71	— 6.59	262	262	376	119	—1600	—587	—0.25
Lamprey	203	201	—12.76	—4.33	— 8.43	250	240	501	167	—2006	—675	—0.25
Mean	201	208	—13.01	—4.37	— 8.64	251	254	523	161	—2041	—690	—0.26

The rotatory dispersions of the investigated collagens are of the simple type over the studied region 365—578 nm and can hence be represented by the one-term Drude equation. The values of λ_c for both native (^{H}) and denatured (^{D}) collagens are nearly equal, mean values 201 nm and 208 nm, and independent of the species. Since the constant A varies with the helix content of proteins, the difference A^H—A^D is an estimate of the helix content and seems to be relatively invariable in the collagens studied.

When the obtained dispersion values were substituted in the Moffitt-Yang equation, the mean values of λ_o were 251 nm for the native and 254 nm for the denatured collagens. For the comparison of helix contents the quantity b_o^{H-D}/a_o^{H-D} was calculated from the values of constants obtained for native (Kb_o^H and Ka_o^H) and denatured (Kb_o^D and Ka_o^D) collagens. Table VIII shows that also the values of this quantity for the various collagens are nearly equal.

To recapitulate the results of the studies on optical rotatory dispersion, the values of λ_c obtained for both native and denatured collagens are nearly equal for all collagens studied regardless of species. The same applies to λ_o. The helix content of collagen does not seem to vary from one species to another.

DENATURATION TEMPERATURES

The denaturation temperatures of dissolved collagens give information only on the intramolecular forces that stabilize the tropocollagen molecule. The thermal shrinkage temperatures give in addition information on the interaction of native tropocollagen molecules.

Table IX presents values of T_D for several citrate-soluble collagens (T_D is defined as the temperature at which a 50 % change has occurred in optical rotation). Mammalian and avian collagens, that is, collagens of homoiothermic animals, have T_D values at least 15° higher than the

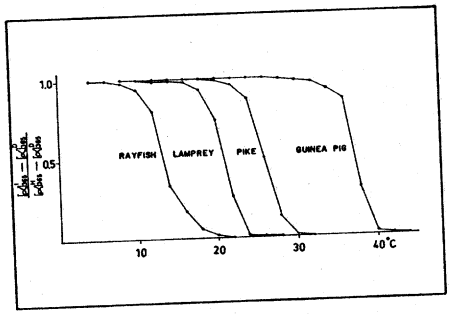


Fig. 4. Denaturation curves of different citrate-soluble collagens.
Abscissa: temperature. *Ordinate*: the proportion of native collagen at temperature t determined from optical rotation.

Table IX.

Denaturation temperatures of collagens in the liquid (T_D) and solid (T_S) state.

The T_D values were determined by polarimetry of solutions of citrate-soluble collagens in a 0.15 M citrate buffer of pH 3.7. The T_S values and the relative contractions of the skins were determined in water.

	T_D	T_S		Shrinkage, percent	
		First	Second	First	Second
Cow	—	70°—73°	—	—	—
Calf	37.4°	68°—71°	—	35—50	—
Guinea pig,					
full-grown	37.1°	—	—	—	—
growing	37.3°	—	—	—	—
Chick	40.7°	64°—67°	—	35—45	—
Frog	25.3°	58°—61°	—	30—35	—
Flounder	20.7°	51°—53°	(72°—76°?)	35—45	(10—25?)
Burbot	19.8°	—	—	—	—
Pike	26.0°	—	—	—	—
Dogfish	25.1°	53°—55°	(65°—75°?)	10—20?	(5—20?)
Rayfish	13.2°	36°—39°	60°—64°	15—35	5—25
Hagfish	15.7°	33°—36°	59°—62°	15—30	(—15)—25
Lamprey	21.0°	44°—46°	65°—68°	3—6	20—25

T_D values of collagens of poikilothermic animals. The denaturation curves in Fig. 4 show that rayfish collagen begins to lose its ordered structure already at temperatures below 10°.

The measured thermal shrinkage temperatures reveal that fish and cyclostome skins have distinctly lower T_S values than the skins of warm-blooded animals (Table IX). Another remarkable property that places

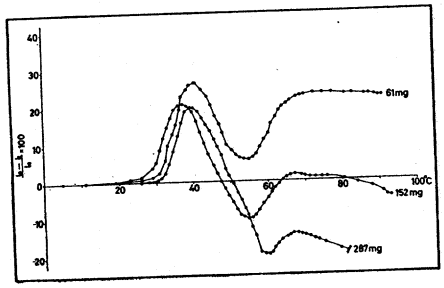


Fig. 5. Biphasic thermal shrinkage curve of hagfish skin loaded with various weights.
Abscissa: temperature. *Ordinate*: shrinkage as a percentage of the original length (l_o) at temperature t.

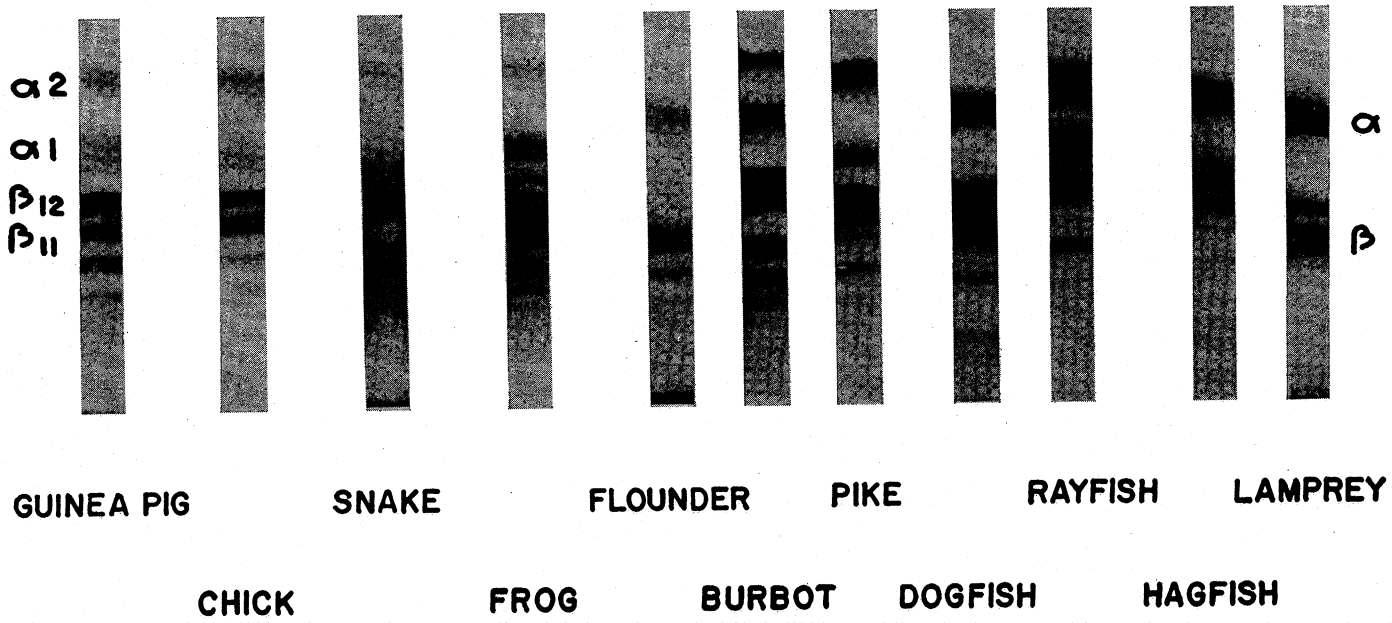


Fig. 6. Starch gel electrophoretic patterns of citrate-soluble collagens from skins of vertebrates. Although there are two α-components in the collagens of bony fishes, there is only one α-component in the collagens of cyclostomes and cartilaginous fishes.

Electrophoretic conditions: gel concentration 14.7 %, sodium acetate buffer of pH 4.7 (I = 0.017), 125 V for 6 h at 38°. Migration upwards.

these aquatic animals in a separate category is the biphasic form of the denaturation curves of their collagens as shown in Fig. 5. Such a curve was obtained for all the studied fish and cyclostome skins except dogfish skin. The extent of the first shrinkage varied from one species to another, but was independent of the applied weight for one animal, but the second shrinkage depended on the weight applied.

The mean differences T_S (1st shrinkage)—T_D and T_S (2nd shrinkage)—T_D are 27° (range 19°—34°) and 48° (range 45°—53°), respectively.

COMPONENTS RESOLVED BY STARCH GEL ELECTROPHORESIS

A general picture of starch gel electrophoretic patterns of citrate-soluble collagens is provided by Fig. 6 where the animals are arranged in phylogenetic order. The figure shows that the electrophoretic pattern is more complex in higher animals and that the evolutionary time interval between cartilaginous and bony fishes means a juncture in the evolution of collagen, flounder being an exception. Although there are two α-components in the collagens of bony fishes, there is only one α-component in the collagens of cyclostomes and cartilaginous fishes.

In order to make sure of the nonidentity of the α-chains, guinea pig collagen was mixed with both hagfish and lamprey collagens and these mixtures were subjected to gel electrophoresis (Figs. 7—8).

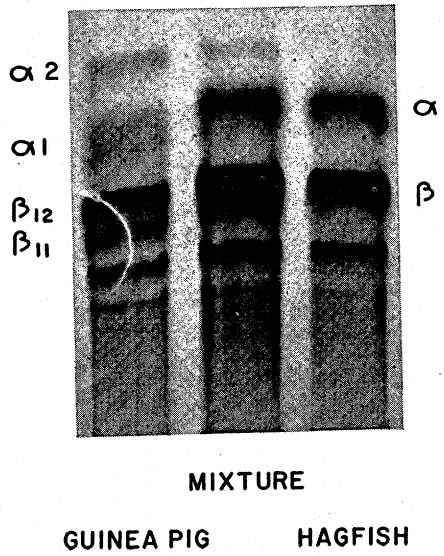
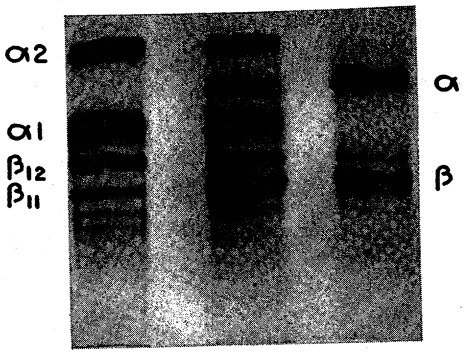


Fig. 7. Starch gel electrophoresis of a mixture of citrate-soluble skin collagens of hagfish and guinea pig.

Electrophoretic conditions: gel concentration 14.7 %, sodium acetate buffer of pH 4.7 (I = 0.017), 125 V for 6 h at 38°.

Fig. 8. Starch gel electrophoresis of a mixture of neutral salt-soluble skin collagens of lamprey and guinea pig.

Electrophoretic conditions: gel concentration 14.7 %, sodium acetate buffer of pH 4.7 (I = 0.017), 125 V for 6 h at 38°.



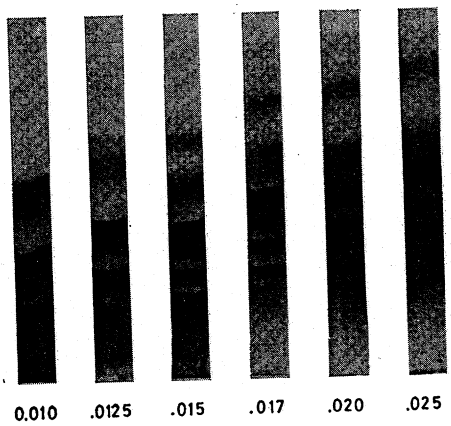


Fig. 9.

Figs. 9—11. Starch gel electrophoretic patterns of citrate-soluble collagen of guinea pig skin when the ionic strength of the buffer was varied.

Fig. 9. Photograph of original patterns.

Fig. 10. Schematic presentation of patterns in Fig. 9.

Abscissa: ionic strength. *Ordinate*: electrophoretic mobility ($cm^2V^{-1}sec^{1-}$). The various shadings refer to the intensities of the individual bands as estimated visually.

Fig. 11. Like the previous figure but the *ordinate* is the relative mobility (the distance moved by the leading edge of the fastest band in each gel was taken as 100).

Electrophoretic conditions: gel concentration 14.7 %, sodium acetate buffer of pH 4.7, 125 V for 6 h at 38°.

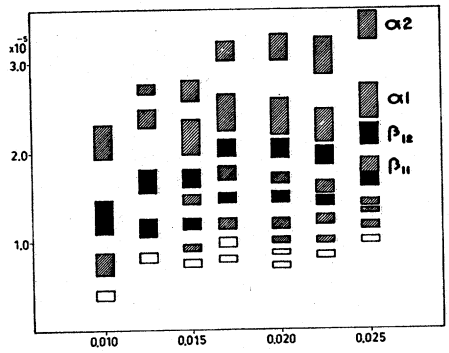


Fig. 10.

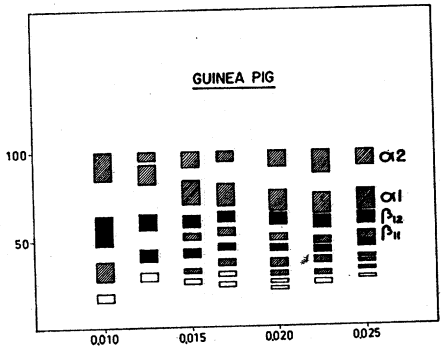


Fig. 11.

The behaviour of various components was studied at different ionic strengths keeping the other conditions unchanged. Fig. 9 presents a photograph of electrophoretic patterns from one series of such experiments. For clarity, this figure was redrawn and the following two figures (Figs. 10—11) show two ways of presenting starch gel electrophoretic patterns.

The citrate-soluble collagens studied can be grouped into three classes (Fig. 25) on the basis of the migration behaviour of their main components as follows. The first class, the so-called guinea pig-type, comprises guinea pig (Figs. 9—11), chick (Fig. 12), frog (Fig. 13), burbot (Fig. 14) and pike collagens (Fig. 15). The α-components and the β-components of the

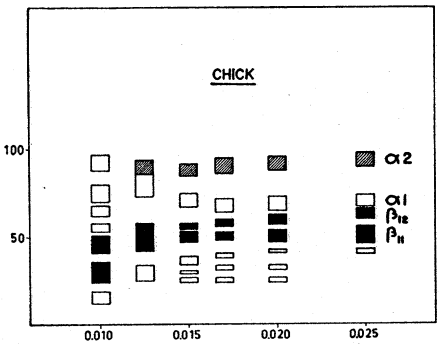


Fig. 12.

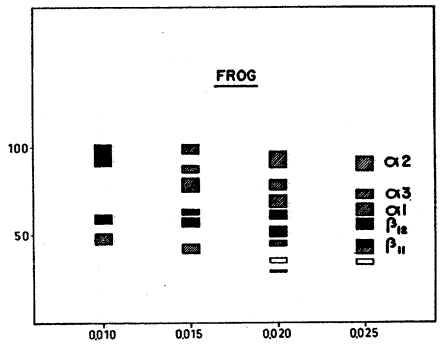


Fig. 13.

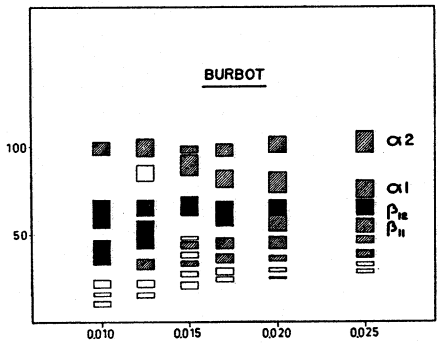


Fig. 14.

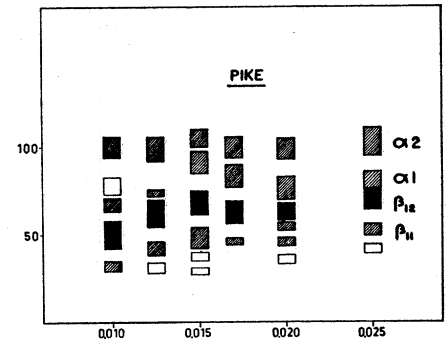


Fig. 15.

Figs. 12—15. Effect of ionic strength on the starch gel electrophoretic patterns of citrate-soluble skin collagens (guinea pig-type) of vertebrates.

The α-components and the β-components of the guinea pig-type collagens are more effectively resolved when the ionic strength is increased.

Abscissa: ionic strength. *Ordinate*: relative mobility.

Electrophoretic conditions: gel concentration 14.7 %, sodium acetate buffer of pH 4.7, 125 V for 6 h at 38°.

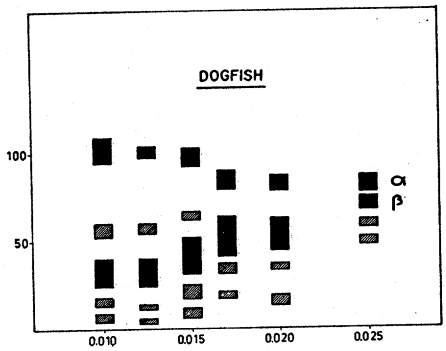


Fig. 16.

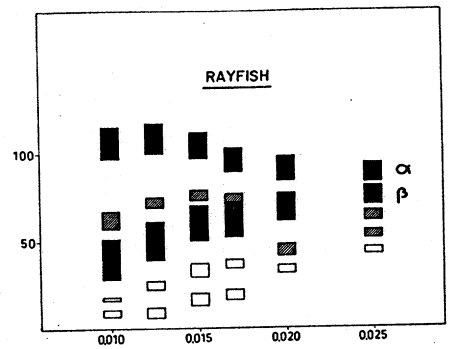


Fig. 17.

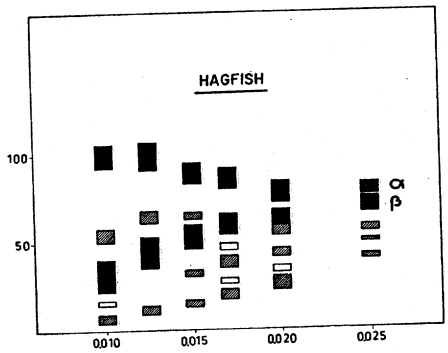


Fig. 18.

Figs. 16—18. Effect of ionic strength on the starch gel electrophoretic patterns of citrate-soluble skin collagens (rayfish-type) of vertebrates.

The mobilities of the α- and β-components of the rayfish-type collagens approach each other when the ionic strength is increased.

Abscissa: ionic strength. *Ordinate*: relative mobility.

Electrophoretic conditions: gel concentration 14.7 %, sodium acetate buffer of pH 4.7, 125 V for 6 h at 38°.

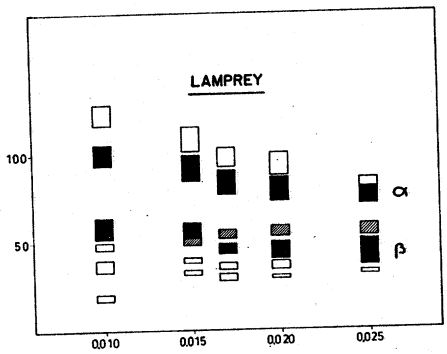


Fig. 19.

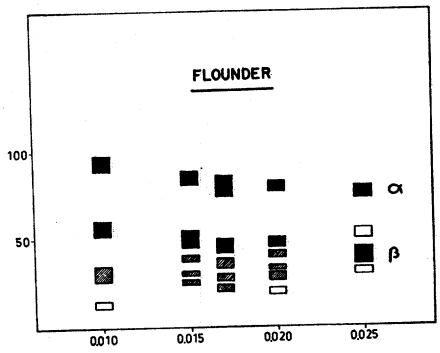


Fig. 20.

Figs. 19—20. Effect of ionic strength on the starch gel electrophoretic patterns of citrate-soluble skin collagens (lamprey-type) of vertebrates.

The mobilities of the α- and β-components of lamprey and flounder collagens vary similarly with ionic strength.

Abscissa: ionic strength. *Ordinate*: relative mobility.

Electrophoretic conditions: gel concentration 14.7 %, sodium acetate buffer of pH 4.7, 125 V for 6 h at 38°.

guinea pig-type collagens are more effectively resolved when the ionic strength is increased. The second class (the rayfish-type) comprises dogfish (Fig. 16), rayfish (Fig. 17) and hagfish collagens (Fig. 18). The mobilities of the α- and β-components of these collagens approach each other when the ionic strength is increased. The third type, the lamprey-type, includes flounder collagen (Fig. 20) in addition to lamprey collagen (Fig. 19). The mobilities of the α- and β-components of these collagens vary similarly with ionic strength.

The effect of pH on the electrophoretic patterns of the three types of collagen resembled the effect of ionic strength as seen in Figs. 21—24. The results of these studies on the effect of ionic strength and pH are summarized schematically in Fig. 25. The α- and β-components of rayfish and lamprey collagens were identified by sedimentation analyses (carried

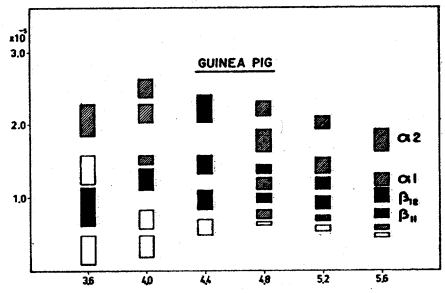


Fig. 21.

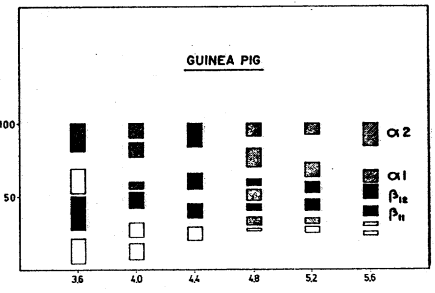


Fig. 22.

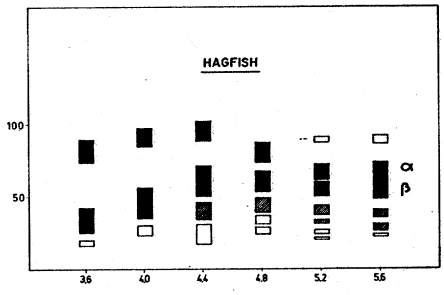


Fig. 23.

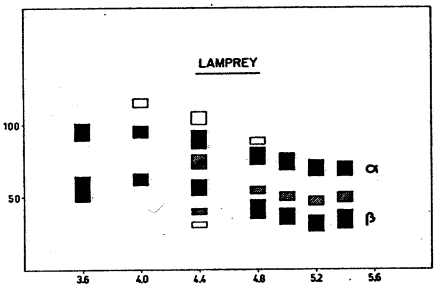


Fig. 24.

Figs. 21—24. Effect of pH on the starch gel electrophoretic patterns of citrate-soluble skin collagens of vertebrates.

The effect of pH on the electrophoretic mobilities of the α- and β-components of the various collagen types resembles the effect of ionic strength.

Abscissa: pH. *Ordinate*: relative mobility in Figs. 22—24 and electrophoretic mobility ($cm^2V^{-1}sec^{-1}$) in Fig. 21.

Electrophoretic conditions: gel concentration 14.7 %, sodium acetate buffer ($I = 0.017$), 125 V for 6 h at 38°.

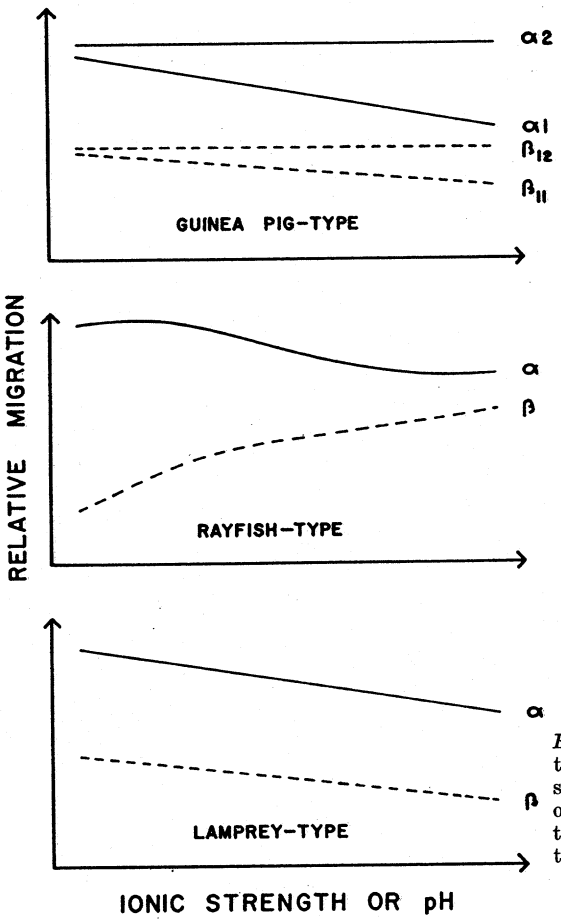


Fig. 25. A schematic representation showing the various types of starch gel electrophoretic patterns of citrate-soluble collagens when the ionic strength or the pH of the buffer was varied.

out by Dr. T. Hollmén) which gave the sedimentation constant ($s^{\circ}_{20,w}$) 4.22S for the faster component and the sedimentation constant 3.04S for the slower component of rayfish collagen and the constants 4.14S and 3.17S for the faster and slower components, respectively, of lamprey collagen.

The appearance of a second and a third new α-component during evolution is discussed later (Fig. 39).

A study of the effect of varying the gel concentration was carried out with guinea pig (Figs. 26—27) and rayfish collagens (Figs. 28—29). The sieve effect which separates the α- and β-components according to their molecular weights is observed with both collagens; this separation is more marked at the lower ionic strength (0.010). The electrophoretic

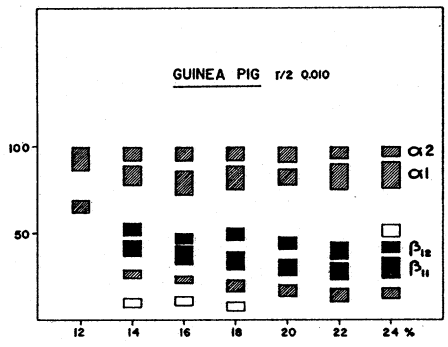


Fig. 26.

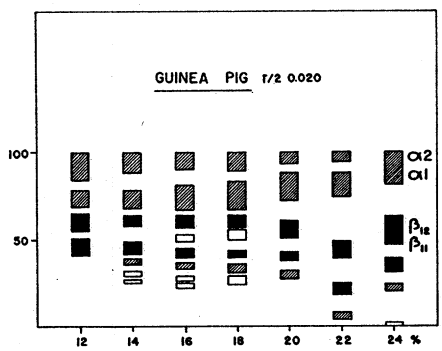


Fig. 27.

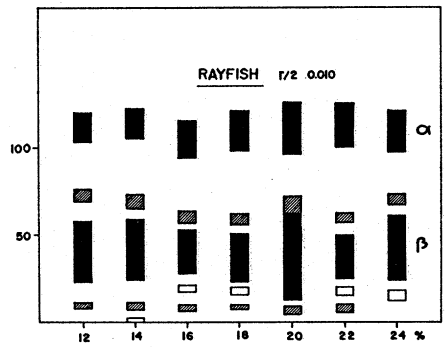


Fig. 28.

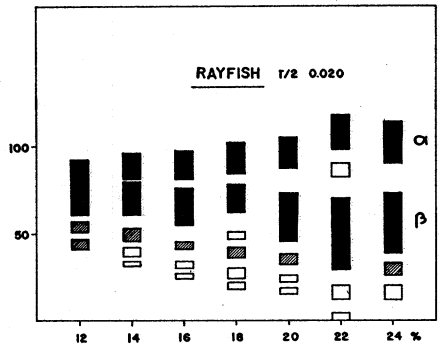


Fig. 29.

Figs. 26—29. Effect of gel concentration on the starch gel electrophoretic patterns of citrate-soluble skin collagens of guinea pig and rayfish.

Abscissa: gel concentration (w/v). *Ordinate*: relative mobility.

Electrophoretic conditions: sodium acetate buffer of pH 4.7 ($I = 0.010$ in Figs. 26 and 28; $I = 0.020$ in Figs. 27 and 29), 125 V for 6 h at 38°.

mobilities of the components decreased with increasing gel concentration but the changes in the relative mobilities differed from those observed when the ionic strength or pH was altered.

COMPONENTS RESOLVED BY CARBOXYMETHYLCELLULOSE COLUMN CHROMATOGRAPHY AND STARCH GEL ELECTROPHORESIS

The citrate-soluble collagens of the different species studied could be divided into two groups on the basis of their patterns in CM-cellulose chromatograms. The first group comprised the guinea pig and rayfish types, which all gave similar fractionation patterns composed of two major

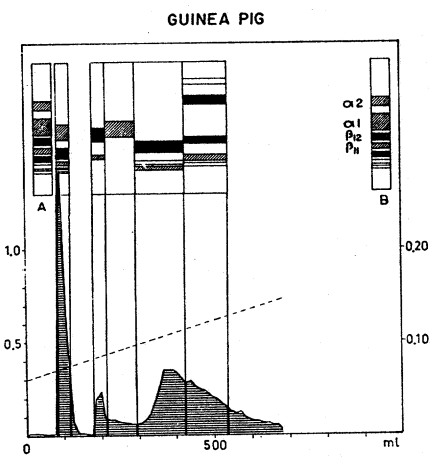


Fig. 30.

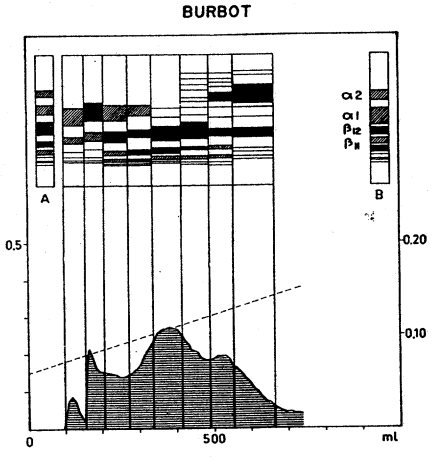


Fig. 31.

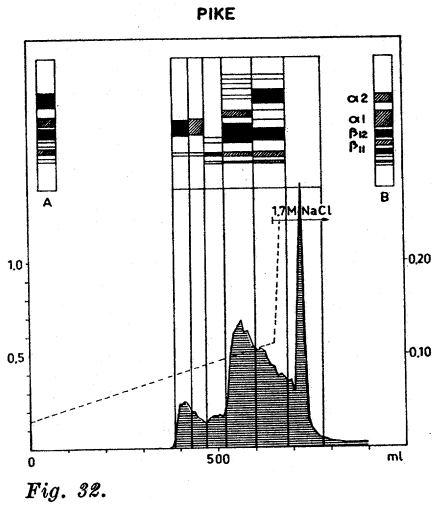


Fig. 32.

Figs. 30—32. CM-cellulose chromatography of citrate-soluble skin collagens (guinea pig-type) of vertebrates and the starch gel electrophoresis of chromatographic fractions.

The α- and β-components of guinea pig-type collagens are eluted from the CM-cellulose column in the order $\alpha 1$, β_{11}, β_{12} and $\alpha 2$.

Abscissa: effluent volume (ml). *Ordinate*: optical density (left) and ionic strength (right). *Solid line*: protein in the effluent, detected with the biuret reaction (absorption at 230 nm in Fig. 31). *Dashed line*: ionic strength of eluting solution. *A*: starch gel electrophoretic pattern before chromatography. *B*: starch gel electrophoretic pattern of citrate-soluble guinea pig collagen. Migration upwards.

peaks with shoulders. The collagens of lamprey and flounder formed the second group.

The first group could be further divided into two subgroups, the guinea pig and rayfish types, by subjecting the chromatographic fractions of collagens to starch gel electrophoresis. The α- and β-components in the guinea pig-type collagens (Figs. 30—32) were eluted from the CM-

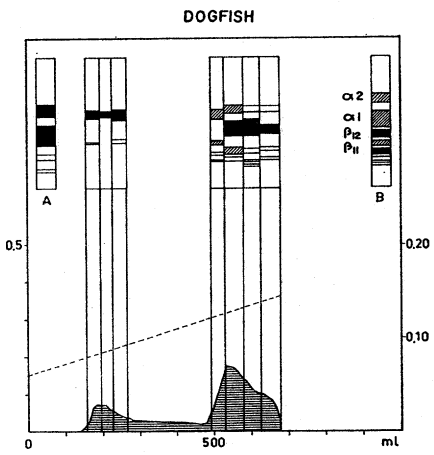


Fig. 33.

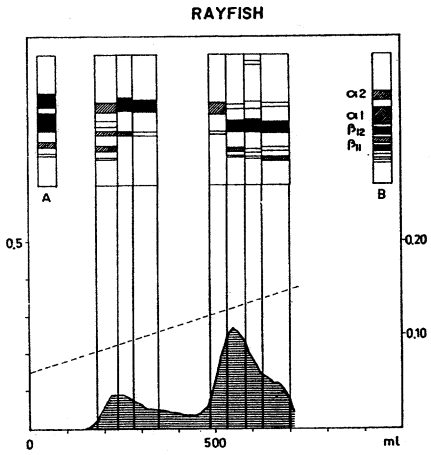


Fig. 34.

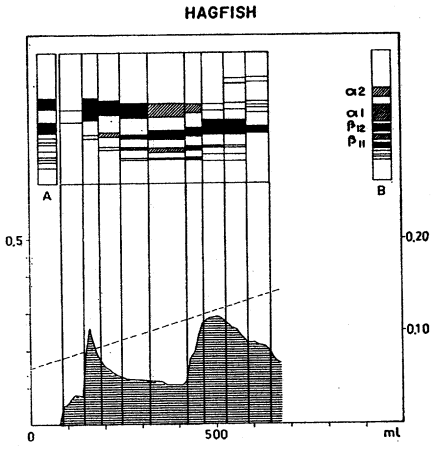


Fig. 35.

Figs. 33—35. CM-cellulose chromatography of citrate-soluble skin collagens (rayfish-type) of vertebrates and the starch gel electrophoresis of chromatographic fractions.

The single α-component of the rayfish-type collagens is eluted before the β-component in CM-cellulose chromatography.

Abscissa: effluent volume (ml). *Ordinate*: optical density (left) and ionic strength (right). *Solid line*: protein in the effluent (absorption at 230 nm). *Dashed line*: ionic strength of eluting solution. *A*: starch gel electrophoretic pattern before chromatography. *B*: starch gel electrophoretic pattern of citrate-soluble guinea pig collagen.

cellulose column in the order α1, β_{11}, β_{12} and α2. The CM-cellulose pattern of the rayfish-type collagen (Figs. 33—35) differs from that of the guinea pig-type in that the α-component and the β-component are more highly resolved. In both types higher aggregates are eluted before the β_{12}- or β-component. The scheme in Fig. 36 shows the behaviour of the α- and β-components of guinea pig-type and rayfish-type collagens in chromatography and gel electrophoresis.

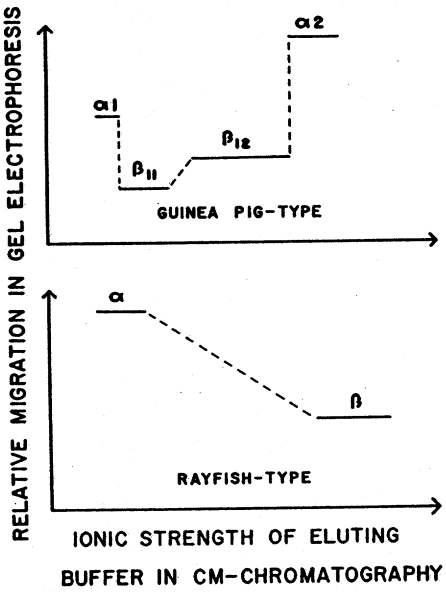


Fig. 36. A schematic representation showing the various types of behaviour of citrate-soluble collagens of guinea pig- and rayfish-types in CM-cellulose chromatography and subsequent starch gel electrophoresis.

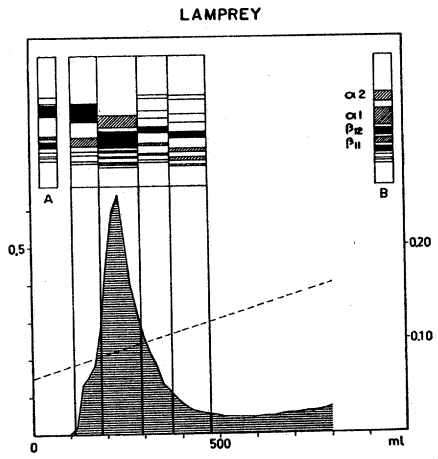


Fig. 37.

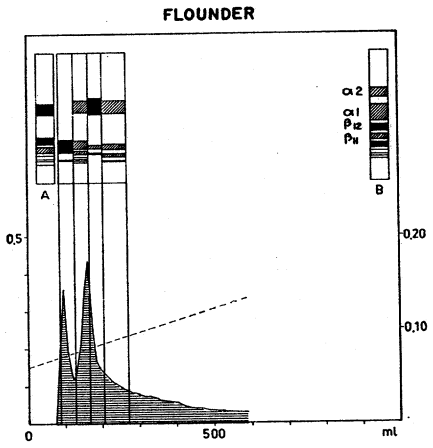


Fig. 38.

Figs. 37—38. CM-cellulose chromatography of citrate-soluble skin collagens (lamprey-type) of vertebrates and the starch gel electrophoresis of chromatographic fractions. *Abscissa*: effluent volume (ml). *Ordinate*: optical density (left) and ionic strength (right). *Solid line*: protein in the effluent (absorption at 230 nm). *Dashed line*: ionic strength of eluting solution. *A*: starch gel electrophoretic pattern before chromatography. *B*: starch gel electrophoretic pattern of citrate-soluble guinea pig collagen.

The lamprey and flounder citrate-soluble collagens of the second group were completely eluted from the CM-cellulose column by an eluting buffer of lower ionic strength than the other collagens. The chromatographic separation of the α- and β-components of lamprey collagen (Fig. 37) was poor, but otherwise this collagen resembled the rayfish-type. Flounder collagen (Fig. 38) differed from lamprey collagen in being more effectively resolved in CM-cellulose chromatography but the components were eluted in the reverse order. No data are available, but the two peaks of flounder collagen may represent components $\alpha 1$ and $\alpha 2$, and not an α-component and a β-component.

SOLUBILITY OF NATIVE AND DENATURED COLLAGENS

Repeated extraction of finely homogenized skin with 0.45 M sodium chloride solution followed by 0.15 M citrate buffer of pH 3.7 at 4° resulted in the dissolution of soluble collagens as shown in Table X. From 30 to

Table X.

Ash, hydroxyproline and soluble collagen contents of vertebrate skins.

	Ash*	Hydroxy-proline**	Percent collagen soluble in	
			neutral salt solution	acid buffer solution
Cow	2.2	9.9	0.4	2.5
Calf	2.3	7.9	1.4	24.4
Guinea pig (full-grown)	2.0	6.9	7.5	17.1
Chick	0.8	2.3	18.0	6.7
Snake	4.5	4.2	0.5	1.3
Frog	6.3	4.6	2.7	4.5
Flounder	1.5	7.3	10.6	13.1
Burbot	0.9	7.9	1.8	46.8
Pike	0.6	7.6	1.2	33.7
Dogfish	26.9	8.4	1.9	44.0
Rayfish	3.7	6.2	0.4	89.5
Hagfish	0.9	8.4	0.5	78.3
Lamprey	0.8	5.6	2.1	74.6

* Ash as a percentage of the weight of the dry skin.
** g/ 100 g of dry, ash-free skin.

90 % of the skin collagens of cyclostomes and fishes but only a few percent of the collagens of a full-grown mammal passed into the citrate solution. A similar difference connected with age was noted on comparing the dissolution of collagens from the skins of a calf and a cow.

The decrease of the solubility of collagen during evolution was reflected in the more facile gelatinization of insoluble cyclostome and fish skin collagens. Table XI shows that more than half of their insoluble collagens dissolved already when the skin residues, after extraction of the soluble collagens, were warmed in 0.01 M sodium acetate buffer of pH 4.8 at 40° for 15 minutes. The corresponding percentages for the insoluble collagens of mammals were less than 10.

Table XI.

Gelatinization of insoluble skin collagens from various vertebrates.

Collagen solubilized in 0.01 M sodium acetate buffer of pH 4.8 at progressively raised temperatures is expressed as a percentage of the original insoluble collagen. The symbols for the various extracts are explained in Fig. 1.

	S 1^{40}	S 2^{65}	S 3^{90}	R 3^{90}	
				S 4^{120}	R 4^{120}
Cow	0.5	6.0	4.9	84.3	4.3
Calf	2.7	63.7	12.3	20.1	1.2
Guinea pig,					
full-grown	9.2	60.6	14.3	15.4	0.5
growing	4.2	85.4	5.8	3.9	0.7
Chick	6.0	52.1	20.4	21.5	
Snake	2.7	5.3	27.0	65.0	
Toad	16.9	38.1	35.6	9.4	
Burbot	69.3	12.6*	11.5	6.6	
Pike	78.4	13.5	4.2	3.9	
Hagfish	55.9	16.1*	10.8	17.2	
Lamprey	89.9	4.4*	3.1	2.6	

* Gelatinized 2 hours at +60°.

DISCUSSION

GENERAL

Collagen is a very old protein, 550 million years old or perhaps older, as judged from its wide distribution in the animal kingdom. The information available on the evolution of collagen obtained from studies of fossils is rather meagre and provides only the age of collagen. It has been established by electron microscopy and X-ray diffraction methods that the oldest collagen fibrils in remnants of bones and teeth are 170—200 million years old (Isaacs *et al.* 1963, Doberenz and Lund 1966). From the amino acid compositions of residues from fossils, Abelson (1963) and Armstrong and Tarlo (1966) concluded that collagen existed in animals that lived 370 million years ago.

Since organic material easily decomposes with time, fossils are not suitable for investigations of the evolution of collagen. Knowledge of the phylogeny of collagen can thus be only derived from existing animals by methods of comparative biochemistry.

PRIMARY STRUCTURE

Because the complete amino acid sequences of collagen chains are not known in detail, no information is available on the primary structures of collagens of different species. The amino acid compositions have, however, been studied by many investigators.

Tables I, II, VI and VII present the relative proportions of individual amino acids and their ranges which show that regardless of the species all collagens are closely similar in amino acid composition. The ratio of *polar* and *nonpolar* amino acids is constant, the latter comprising about two thirds of all amino acids. The invariable content of glycine, one third of the total, is a condition for the triple helical conformation. The proportion of *acidic* and *basic* amino acids is rather constant although large variations occur in the proportions of lysine, hydroxylysine and histidine. The total number of lysine and hydroxylysine residues is constant except in hagfish collagen. The great variation in histidine content may only be

apparent and due to the low proportion present. The number of *hydroxyl groups* remains constant even though the proportions of serine, threonine and hydroxyproline vary greatly. The significance of the variation of hydroxyl groups and *imino acids* will be discussed later. The only *sulphur-containing* amino acid in vertebrate collagens is methionine.

Comparisons of amino acid compositions of collagens of different species show that amphibian, fish and cyclostome collagens contain less imino acids than collagens of higher vertebrates. Eastoe (1957) proposed that the low proportion of hydroxyproline in fish collagens is compensated for by a high proportion of aliphatic hydroxy amino acids. Like the contents of serine and threonine the content of methionine decreases with evolution.

Since the amino acid sequence of collagen is unknown, phylogenetic comparisons of the primary structures of collagens of different species cannot be carried out as in the case of haemoglobin (Buettner-Janusch and Hill 1965), cytochrome *c* (Margoliash 1963) and fibrinopeptides (Blombäck, Doolittle and Blombäck 1965). Therefore only the amino acid compositions can be compared. The sums of the differences between the relative numbers of amino acid residues in collagens of different species given in Table VI can be compared in Table XII. To eliminate errors

Table XII.
Differences between the amino acid compositions of citrate-soluble collagens given in Table VI.

	Pig	Chick	Frog	Flounder	Dogfish	Rayfish	Hagfish
Chick	30.5						
Frog	82.5	71					
Flounder	97.5	85	31				
Dogfish	63.5	57	58	67			
Rayfish	119	107	90	91	54		
Hagfish	102.5	113	51.5	36	85	85	
Lamprey	82.5	69	26	30	47	83	48

arising from possible differences in hydroxylation, the proportions of imino acids and the proportions of lysines were taken instead of the proportions of the individual amino acids. The figures in Table XII do not reveal two consecutive changes of primary structure that did not lead to an altered amino acid composition. In general, however, the differences in the amino acid compositions are related to the evolutionary ages of the species. The amino acid composition of lamprey collagen is an exception and differs, as has already been mentioned, from the amino acid composition of hagfish collagen.

The polar regions along the tropocollagen molecule can be located by means of the typical cross-striated pattern of its segment-long-spacing collagen. Great similarities are observed on comparing SLS collagens of the present investigation and the pattern of squid SLS collagen (Pikkarainen *et al.* 1966, unpublished work by the author in Prof. Kühn's laboratory in 1967). Quite recently, Nordwig and Hayduk (1967) did not find any differences between the SLS patterns of collagens of *Actinia equina* L., a sea anemone, and a calf.

SECONDARY STRUCTURE

A triple helical conformation in crystalline regions is common to all collagens. Regardless of the vertebrate species, the helix content was constant in the citrate-soluble collagens of the present investigation. This was shown by the approximate constancy of the differences between the specific rotations of native and denatured collagens as well as the constancy of the quantities $A^H—A^D$ and b_0^{H-D}/a_0^{H-D}.

The functional evolution of collagen is characterized by an increase in structural stability. This is experimentally observed as a higher denaturation temperature of the collagen. In the present study the stabilities of collagens to heat measured in both the solid and the liquid state show that the cold-blooded animals clearly differ from warm-blooded animals in having a weaker collagen structure. A comparison of either the T_S or the T_D values (Tables IV and IX) reveals that although an evolutionary increase in the stability of collagen has occurred, a large difference exists between various species of the same class, *e.g.*, between dogfish and rayfish, and between hagfish and lamprey. The material is too small to permit one to draw any conclusions about the natural selection of mutations in the evolution of collagen stability. The apparent differences between the

T_D values of rayfish (13°) and smooth dogfish (25°) determined in the present investigation and the T_D value of spiny dogfish (16°) reported by Lewis and Piez (1961, 1964) could be due to an influence of dissimilar environments on closely related species.

In conformity with recent views, the effects of cross-links, van der Waals forces, and the hydroxyl groups of hydroxyproline are of no importance for the stability of the collagen molecule. Significant in this respect are not only the numbers of hydrogen bonds and pyrrolidine rings, that is, the contents of proline and hydroxyproline, but also the distribution of these amino acids throughout the molecule (Josse *et al.* 1964, Rao and Harrington 1966).

The constancy of the number of hydroxyl groups is characteristic of all collagens of mesodermal origin. The contents of serine, threonine and hydroxyproline found in the present material can be compared in Table XIII which shows that changes in the number of serine and threonine residues are compensated for by changes in the number of hydroxyproline residues (even though the variations in the proportions of tyrosine and hydroxylysine are large, they do not significantly influence the total number of hydroxyl groups and can be neglected). Proline and lysine are believed to be hydroxylated in the peptide chain to the corresponding hydroxy amino acids. The codons of proline, threonine and serine, besides being degenerate, differ only in the first "letter". When these amino acids are in peptide chains, the hydroxyl groups of serine and threonine are

Table XIII.

Comparison of hydroxyproline, threonine and serine contents of citrate-soluble collagens. The collagens are listed in the order of increasing stability, T_D (Table IX). The proportion of each amino acid is expressed as the number of residues per 1000 residues.

	Hydroxy-proline	Threonine	Serine	Hydroxy amino acids	Imino acids
Rayfish	61	30	71	170	145
Hagfish	62	27	71	165	154
Flounder	63	20	65	152	166
Lamprey	66	23	58	152	173
Dogfish	72	25	46	146	171
Frog	65	22	56	148	167
Chick	99	19	27	148	212
Pig*	91	18	35	154	223

* Pig-skin gelatin, see p. 22.

stereochemically identical whereas the hydroxyl group of hydroxyproline deviates slightly from these. Replacement of serine by threonine or *vice versa* in the chain is thus not accompanied by a change in the hydrogen bond energy of the hydroxyl group as would be in the case if either were replaced by hydroxyproline.

The way in which hydroxyl groups stabilize the collagen structure is unknown. Combined O- and N-acetylation diminishes the T_s value of calf skin collagen from 64°—66° to 40°—44°, while N-acetylation alone has no effect (Gustafson 1954b). If we accept the Rich-Crick type II model of collagen structure, the hydrogen bond formed by hydroxyproline is intermolecular (intramolecular in type I). The investigations of Harrington (1964), Bensusan and Nielsen (1964) and Rao and Harrington (1966) support the Madras structure with two interchain hydrogen bonds to every amino acid triplet (only one hydrogen bond in the Rich-Crick types I and II). One hydrogen bond per triplet is, however, enough to produce the triple helical conformation as demonstrated by the studies of Rogulenkova, Millionova and Andreeva (1964) and Traub and Yonath (1966) on the synthetic polypeptides poly(Gly-Pro-Hypro) and poly(Pro-Gly-Pro). Since the ordered structure is found in limited regions throughout the molecule, it is not impossible that both structures, those with one and two stabilizing hydrogen bonds to each amino acid triplet, should be taken into consideration.

The higher heat stability of the secondary structure of mammalian and avian collagens compared to that of lower vertebrate collagens leads to speculations about the connections between imino acid, serine and threonine contents and high body temperatures of higher vertebrates and homoiothermy in general.

TERTIARY STRUCTURE

The citrate-soluble collagens examined in the present study were separated into three types by gel electrophoresis and CM-cellulose chromatography. In gel electrophoresis the separation is determined by both the charges and the sizes of the particles, whereas the charge is the principal resolving property in CM-cellulose chromatography.

At constant ionic strength the electrophoretic mobility is directly proportional to the particle charge. The thickness of the ionic double layer surrounding and the hydrodynamic volume taken up by the particle are

constant. The experiments performed to determine the effect of a change in ionic strength gave gel electrophoretic patterns similar to those obtained when the pH was varied. Jackson and Neuberger (1957) have shown that an increase in the ionic strength of a collagen solution leads to a decrease in the isoelectrophoretic point of the collagen. This has been attributed mainly to increased binding of anions. Another consequence of an increase in ionic strength is that the hydrodynamic volume is reduced and the ionic double layer moves in towards the particle and alters the net charge of the particle (Bull 1964).

Comparison of the behaviour of various α-chains in Figs. 9—25 reveals that the mobility of the $\alpha 2$-chain is highly dependent on ionic strength and pH (Figs. 9—10), while the mobilities of the $\alpha 1$- and α-chains (rayfish-type) resemble each other in being independent of these two parameters. The similarity of chains $\alpha 1$ and α was further confirmed in the experiments where the effect of gel concentration was studied (Figs. 27 and 29) and in the gel electrophoresis of collagen mixtures (Fig. 7). The influence of ionic strength or pH on migration is greater for the β-component of the rayfish-type collagen than for the β-component of the guinea pig-type.

Investigations on the influence of gel concentration on electrophoretic mobility revealed that the α- and β-components (which differ in molecular weight) behaved differently when the ionic strength was changed at the same gel concentration (Figs. 26—29). This can be explained by assuming that the decrease in ionic strength has a greater effect on the hydrodynamic volume of the particle than on its charge. The effect of gel concentration on the migration of chains $\alpha 2$ and $\alpha 1$ of guinea pig and the chain α of rayfish collagen (which chains are of equal molecular weight) at ionic strength 0.020 resembled the effect of a decrease in either ionic strength or pH (Fig. 25). Since starch is known to contain charged groups, the explanation could be a change in the ionic strength resulting from the variation in gel concentration.

Lamprey collagen clearly occupies a special position on the basis of its chain composition and chain behaviour when compared to other collagens. This exceptional position is confirmed by amino acid analyses. Another exception is flounder collagen. Analyses of collagens of other flatfishes living in the same kind of environment might possibly shed more light on this finding.

The appearance of the bony fishes seems to have been a turning point in the development of collagen, perhaps because the collagen became more easily ossified. On the basis of the present results, the following hypothesis of the evolution of α-chains is proposed (Fig. 39). The primitive collagen

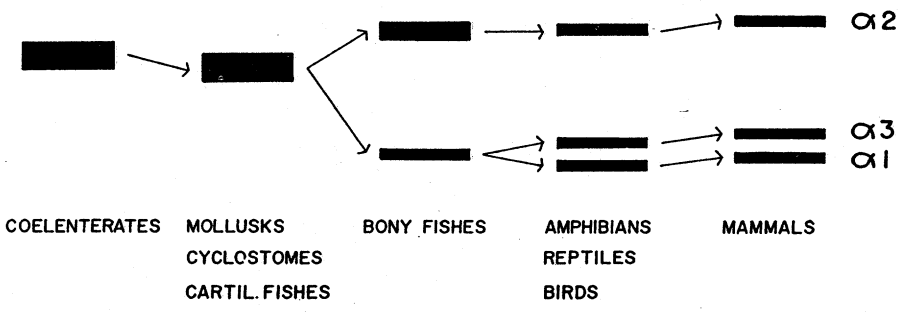


Fig. 39.

molecule was composed of three identical α-chains. Possibly as a result of two successive duplications of the "collagen gene" three α-chains developed which underwent independent mutations leading to the three different α-chains of the higher vertebrates (Pikkarainen and Kulonen 1967).

QUATERNARY STRUCTURE

The collagen fibres in tissues are formed from tropocollagen molecules which aggregate, according to recent views, side to side and overlap a quarter of their length. This model is not, however, generally accepted (*e.g.* Grant, Horne and Cox 1965). The periodicity of 640/700 Å observed in native collagen fibre in electron microscopy is related to the mode of aggregation. The absence of periodicity in native fibres of *Lumbricus* and *Ascaris* collagens and of vitrosin is ascribed to the fact that the basic molecule is not tropocollagen, but a polymer of this (Maser and Rice 1963a, Beer, Josse and Harrington 1964 and Olsen 1965).

The solubility of collagen reflects the stability of the quaternary structure. Interspecies comparisons have shown (Table X) that up to ninety per cent of the native collagens in cyclostomes and fishes is soluble while the corresponding proportion for native collagens of full-grown higher vertebrates is only a few per cent. The increase in stability during evolution is reflected also in the ease of gelatinization of the insoluble collagen (Table XI).

An increase in the intermolecular attractive forces, perhaps an increase in the number of cross-links, could be the reason for the decreased solu-

bility. The histological differences, laminated fibre structure *versus* three-dimensional, cannot alone explain the differences in solubility. Parallel fibres are found in mammalian achilles tendon without the solubility being high. There may also exist differences between mammalian and the more primitive fish collagens in their ability to form stable aggregates.

As shown in Table X, a decrease in the solubility of collagen occurs in mammals with age. It might thus be possible to study the process of aging by comparing animals that differ in evolutionary age if this age-dependent change in collagen solubility could be taken as a recapitulation.

The existence of the biphasic denaturation curve, which was a constant finding in the determinations of shrinkage temperatures of rayfish, hagfish and lamprey skins, could be explained by assuming the presence of two types of chemical bond that differ in stability. In these animals the second shrinkage was abrupt in contrast to a very slow shrinkage of flounder and dogfish skins. The two-stage shrinkage is in agreement with the two-stage denaturation curve of collagen solutions (Engel 1961). Rigby (1961, 1967) was able to show that thermal shrinkage proceeded in two stages also in urea, dilute hydrochloric acid and dilute sodium hydroxide solutions and that the first temperature T_T coincided with T_D.

CONCLUDING REMARKS

As a concluding remark, the author would like to put forward the following hypothesis of the evolution of collagen. The basic molecule, tropocollagen, is in the primitive form composed of three identical peptide chains in triple helical conformation. This structure has developed into another that differs from the primitive structure by having all chains dissimilar, probably as a result of repeated duplications of the "collagen gene" followed by independent changes in the DNA corresponding to each chain. Mutations involving single amino acid replacements have been permitted as long as they have not given rise to changes in the tertiary structure of collagen.

An essential feature in the evolution of collagen seems to have been the partial replacement of serine and threonine by proline (because of the close similarity of their codons) which is converted to hydroxyproline. The structure of collagen evidently presupposes a certain fairly constant content of hydroxyl groups. Thus, the contents of imino acids, hydroxyproline, serine and threonine all correlate with the stability of collagen, the last two contents, however, negatively.

The lower solubility of collagens of higher animals at both low and high temperatures is due to more close-packed arrangements of tropocollagen molecules and represents development occurring at the level of the quaternary structure of collagen. This same phenomenon is observed also in the development of an individual.

SUMMARY

The aim of the present investigation was to obtain more information on the phylogeny of collagen and to define the essential features of collagen structure. Various fractions of collagen were isolated from the skins of the following 12 taxonomically widely differing species representing all vertebrate classes: cow and guinea pig (mammals), chick (birds), snake (reptiles), frog (amphibians), flounder, burbot and pike (bony fishes), smooth dogfish and rayfish (cartilaginous fishes), and hagfish and lamprey (cyclostomes). The skin collagens were studied by a variety of methods and information was obtained about the various levels of structural organization.

1. Features common to all collagens were the constant total contents of nonpolar amino acids (two thirds; one half of which is glycine) and acidic amino acids as well as the constant number of hydroxyl groups (Table VI).

An evolutionary trend was detected in the contents of methionine, serine and threonine which decreased and in the contents of hydroxyproline and proline which increased. Dogfish and rayfish collagens had higher contents of isoleucine and arginine than the other collagens. The relative number of glutamic acid, valine and leucine residues in these cartilaginous fishes was clearly higher than in flounder and cyclostomes and about as high as in mammals. Of the collagens investigated, cyclostome collagens had the lowest content of leucine. Lamprey collagen resembled the collagens of higher vertebrates in its content of hydroxy amino acids.

In general, the amino acid compositions of the collagens of the different species were related to the evolutionary ages of the species (Table XII).

2. The optical rotations of collagens were determined at different wavelengths. By fitting the equations of Drude and Moffitt-Yang to the data, the helix content was found to be nearly the same in all the collagens studied (Table VIII).

The stability of collagen was studied by determining thermal shrinkage temperatures (T_S) and denaturation temperatures in solution (T_D) (Table IX). Homoiothermal vertebrates had higher denaturation temperatures than poikilothermal. In the determination of T_S, the skins of rayfish,

hagfish and lamprey were found to give a biphasic denaturation curve (Fig. 5), that is, after initial contraction, the skin stretched and then underwent a second shrinkage. When the T_D values were substracted from the corresponding T_S values, the mean differences were 27° (range 19°—34°) and 48° (range 45°—53°) for the lower and higher T_S values, respectively.

The correlation between chemical structure and stability of collagens is discussed by paying special attention to the significance of hydroxyl groups and imino acids.

3. The component compositions of citrate-soluble collagens were studied by starch gel electrophoresis and CM-cellulose chromatography. The gel electrophoretic patterns of lamprey, hagfish, rayfish and dogfish collagens differed clearly from those of the other collagens (Figs. 9—29). On the basis of the behaviour of various components in gel electrophoresis when the ionic strength or pH was changed under otherwise identical conditions, the collagens were separated into three different groups, a so-called lamprey-type, (lamprey, flounder), a rayfish-type (rayfish, dogfish hagfish) and a guinea pig-type (guinea pig, cow, chick, frog, burbot, pike). The collagens were allocated to the same three groups also on the basis of their behaviour on chromatographic fractionation on CM-cellulose followed by starch gel electrophoresis of the obtained fractions (Figs. 30—38).

4. Finely homogenized skins were extracted with 0.45 M sodium chloride solution followed by extraction with 0.15 M citrate buffer of pH 3.7 at 4°. From 30 to 90 percent of the skin collagens of lamprey, hagfish, rayfish, dogfish, pike and burbot was soluble in the citrate buffer solution (Table X). The high solubility of these collagens was also reflected in the facile gelatinization of their insoluble collagens at low temperatures (Table XI).

5. The following hypothesis of the evolution of collagen is put forward. The triple helical collagen molecule composed of three different α-chains has developed from a primitive form which was composed of three identical α-chains (Fig. 39). The structural evolution has led to increased functional stability of the collagen at the levels of both secondary (thermal stability) and quaternary structures (insolubility in solvents).

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REFERENCES

ABELSON, P. H., Paleobiochemistry. In: Oparin, A. I. (ed.), *Evolutionary Biochemistry. Proc. V. int. Congr. Biochem. Moscow,* New York, Macmillan Co. 1963. *3.* 52—68.

ALTGELT, K., A. J. HODGE and F. O. SCHMITT, Gamma tropocollagen: A reversibly denaturable collagen macromolecule. *Proc. nat. Acad. Sci.* (Wash.) 1961. *47.* 1914—1924.

ARMSTRONG, W. G. and L. B. H. TARLO, Amino-acid components in fossil calcified tissues. *Nature* (Lond.) 1966. *210.* 481—482.

ASTRUP, H. N., A. M. MARKO and E. G. YOUNG, Some physical and chemical properties of acid-soluble ichthyocol. In: Stainsby, G. (ed.), *Recent Advances in Gelatin and Glue Research.* London, Pergamon Press 1958. 76—81.

BAKERMAN, S., Physical parameters of citric acid soluble human skin collagen of the newborn. *J. Gerontol.* 1961a. *16.* 388.

BAKERMAN, S., Thermal denaturation of citric acid soluble human skin collagen of the newborn. *Fed. Proc.* 1961b. *20.* 382.

BAKERMAN, S., Distribution of the α- and β-components in human-skin collagen with age. *Biochim. biophys. Acta* (Amst.) 1964. *90.* 621—623.

BEER, J. JOSSE and W. F. HARRINGTON, (unpublished results), cited by Josse, J. and W. F. Harrington, Role of pyrrolidine residues in the structure and stabilization of collagen. *J. molec. Biol.* 1964. *9.* 269—287.

BENSUSAN, H. B. and S. O. NIELSEN, The deuterium exchange of peptide group hydrogen atoms during the gelatin→ collagen-fold transition. *Biochemistry* 1964. *3.* 1367—1377.

BERENDSEN, H. J. C. and C. MIGCHELSEN, Hydration structure of collagen and influence of salts. *Fed. Proc.* 1966. *25.* 998—1002.

BEVERIDGE, J. M. R. and C. C. LUCAS, Amino acids of isinglass. *J. biol. Chem.* 1944. *155.* 547—556.

BLOMBÄCK, B., R. F. DOOLITTLE and M. BLOMBÄCK, Fibrinogen: structure and evolution. In: Peeters, H. (ed.), *Protides of the Biological Fluids.* Amsterdam, Elsevier Publ. Co. 1965. *12.* 87—94.

BOEDTKER, H. and P. DOTY, The native and denatured states of soluble collagen. *J. Amer. chem. Soc.* 1956. *78.* 4267—4280.

BORASKY, R., Collagens — — phylogenetic considerations. *Physiol. bohemoslov.* 1965. *14.* 206—213.

BORNSTEIN, P., G. R. MARTIN and K. A. PIEZ, Intermolecular cross-linking of collagen and the identification of a new beta-component. *Science* 1964. *144.* 1220—1221.

BORNSTEIN, P. and K. A. PIEZ, A biochemical study of human skin collagen and the relation between intra- and intermolecular cross-linking. *J. clin. Invest.* 1964. *43.* 1813—1823.

BOWES, J. H., R. G. ELLIOTT and J. A. MOSS, The extraction of soluble protein from skin by alkaline solutions. In: Stainsby, G. (ed.), *Recent Advances in Gelatin and Glue Research.* London, Pergamon Press 1958. 71—75.

BUETTNER-JANUSCH, J. and R. L. HILL, Evolution of hemoglobin in primates. In: Bryson, V. and H. J. Vogel (eds.), *Evolving Genes and Proteins.* New York, Academic Press 1965. 167—181.

BULL, H. B., *An Introduction to Physical Biochemistry*. Philadelphia, F. A. Davis Company 1964. p. 320.

BURGE, R. E. and R. D. HYNES, The optical rotatory power of collagen. *Nature* (Lond.) 1959a. *184*. 1562—1563.

BURGE, R. E. and R. D. HYNES, The thermal denaturation of collagen in solution and its structural implications. *J. molec. Biol.* 1959b. *1*. 155—164.

CADAVID, N. G., B. DENDUCHIS and R. E. MANCINI, Soluble collagens in normal rat skin, from embryo to adulthood. *Lab. Invest.* 1963. *12*. 598—605.

CLAUSEN, B., Influence of age on connective tissue. Hexosamine and hydroxyproline in human aorta, myocardium, and skin. *Lab. Invest.* 1962. *11*. 229—234.

COHEN, C., Optical rotation and helical polypeptide chain configuration in collagen and gelatin. *J. biophys. biochem. Cytol.* 1955. *1*. 203—214.

COMTE, P., (ed.), *Biochimie et Physiologie du Tissu Conjonctif*. Lyon 1966. pp. 700.

COOPER, D. R. and R. J. DAVIDSON, The effect of ultraviolet irradiation on soluble collagen. *Biochem. J.* 1965. *97*. 139—147.

CROSBY, N. T. and G. STAINSBY, The thermal stability of dissolved collagens. *Research* 1962. *15*. 427—435.

DAVIS, G. A., R. J. SANTEN and B. W. AGRANOFF, The production of a linear density gradient by means of a proportioning pump. *Analyt. Biochem.* 1965. *11*. 153—154.

DICKERSON, J. W. T. and P. M. V. JOHN, The effect of sex and site on the composition of skin in the rat and mouse. *Biochem. J.* 1964. *92*. 364—368.

DIXON, G. H., Mechanisms of protein evolution. In: Campbell, P. N. and G. D. Greville (eds.), *Essays in Biochemistry*. London, Academic Press 1966. *2*. 147—204.

DOBERENZ, A. R. and R. LUND, Evidence for collagen in a fossil of the lower Jurassic. *Nature* (Lond.) 1966. *212*. 1502—1503.

DOTY, P. and T. NISHIHARA, The molecular properties and thermal stability of soluble collagen. In: Stainsby, G. (ed.), *Recent Advances in Gelatin and Glue Research*. London, Pergamon Press 1958. 92—99.

DRAKE, M. P., P. F. DAVISON, S. BUMP and F. O. SCHMITT, Action of proteolytic enzymes on tropocollagen and insoluble collagen. *Biochemistry* 1966. *5*. 301—312.

EASTOE, J. E., The amino acid composition of mammalian collagen and gelatin. *Biochem. J.* 1955. *61*. 589—600.

EASTOE, J. E., The amino acid composition of fish collagen and gelatin. *Biochem. J.* 1957. *65*. 363—368.

EASTOE, J. E., A semi-micro method for the determination of amino acids by ion-exchange chromatography. *Biochem. J.* 1961. *79*. 652—656.

EASTOE, J. E. and A. A. LEACH, A survey of recent work on the amino acid composition of vertebrate collagen and gelatin. In: Stainsby, G. (ed.), *Recent Advances in Gelatin and Glue Research*. London, Pergamon Press 1958. 173—178.

EDDS, JR., M. V. (ed.), *Macromolecular Complexes*. New York, The Ronald Press Company 1961. pp. 257.

ENGEL, J., Untersuchung des Mechanismus der Denaturierung und Renaturierung von löslichem Kollagen mit der Lichtstreungsmethode. *Hoppe-Seylers Z. physiol. Chem.* 1961. *325*. 287—288.

ENGEL, J. and G. BEIER, Vergleich der molekularen Daten von Tropokollagen verschiedener Kalbshäute im nativen und denaturierten Zustand. *Hoppe-Seylers Z. physiol. Chem.* 1963. *334*. 201—214.

ESIPOVA, N. G., On the character of certain hydrogen bonds in collagen. *Biofizika* 1957. *2*. 461—464.

FASMAN, G. D., Optical rotatory dispersion. In: Colowick, S. P. and N. O. Kaplan (eds.), *Methods in Enzymology*. New York, Academic Press 1963. *6*. 928—957.

FLORKIN, M., On the phylogeny of proteins. In: Peeters, H. (ed.), *Protides of the Biological Fluids*. Amsterdam, Elsevier Publ. Co. 1965. *12*. 17—28.

FLORKIN, M., *A Molecular Approach to Phylogeny*. Amsterdam, Elsevier Publ. Co. 1966. pp. 176.

FLORY, P. J. and R. R. GARRETT, Phase transitions in collagen and gelatin systems. *J. Amer. chem. Soc.* 1958. *80*. 4836—4845.

FLORY, P. J. and E. S. WEAVER, Helix $\rightleftarrows$ coil transitions in dilute aqueous collagen solutions. *J. Amer. chem. Soc.* 1960. *82*. 4518—4525.

FRANCOIS, C. J. and M. J. GLIMCHER, The isolation of the α chains of bone collagen. In: Comte, P. (ed.), *Biochimie et Physiologie du Tissu Conjonctif*. Lyon 1966. 513—520.

FUJIMORI, E., Ultraviolet light irradiated collagen macromolecules. *Biochemistry* 1966. *5*. 1034—1040.

GRANT, R. A., R. W. HORNE and R. W. COX, New model for the tropocollagen macromolecule and its mode of aggregation. *Nature* (Lond.) 1965. *207*. 822—824.

GRASSMANN, W., Kollagen und Bindegewebe. *Svensk kem. Tidskr.* 1960. *72*. 275—302.

GRASSMANN, W., J. ENGEL, K. HANNIG, H. HÖRMANN, K. KÜHN and A. NORDWIG, Kollagen. In: Zechmeister, L. (ed.), *Fortschritte der Chemie organischer Naturstoffe*. Wien, Springer Verlag 1965. *23*. 195—314.

GRASSMANN, W., K. HANNIG and J. ENGEL, Das quantitative Verhältnis zwischen- α- und β-Komponente des denaturierten, löslichen Kollagens in der Ultrazentrifuge sowie Beschreibung einer schneller sedimentierenden γ-Komponente. *Hoppe-Seylers Z. physiol. Chem.* 1961a. *324*. 284—288.

GRASSMANN, W., K. HANNIG and M. SCHLEYER, Zur Aminosäuresequenzen des Kollagens, II. *Hoppe-Seylers Z. physiol. Chem.* 1960. *322*. 71—95.

GRASSMANN, W., A. NORDWIG and H. HÖRMANN, Aminosäuresequenzen des Kollagens, III. Über den Bau der apolaren Bereiche der Kollagenfaser; Abbau mit Kollagenase. *Hoppe-Seylers Z. physiol. Chem.* 1961b. *323*. 48—60.

GROSS, J., Studies on the formation of collagen I. Properties and fractionation of neutral salt extracts of normal guinea pig connective tissue. *J. exp. Med.* 1958a *107*. 247—263,

GROSS, J., Studies on the formation of collagen II. The influence of growth rate on neutral salt extracts of guinea pig dermis. *J. exp. Med.* 1958b. *107*. 265—277.

GROSS, J., Comparative biochemistry of collagen. In: Florkin, M. and H. S. Mason (eds.), *Comparative Biochemistry. A Comprehensive Treatise*. New York, Academic Press 1963. *5*. 307—346.

GROSS, J. and Y. NAGAI, Specific degradation of the collagen molecule by tadpole collagenolytic enzyme. *Proc. nat. Acad. Sci.* (Wash.) 1965. *54*. 1197—1204.

GUSTAVSON, K. H., The directing influence of the organization of proteins upon their reactivity. *Svensk kem. Tidskr.* 1942. *54*. 74—83.

GUSTAVSON, K. H., The effect of anionic detergents on collagens of mammals and teleostei. *Acta chem. scand.* 1950. *4*. 1171—1184.

GUSTAVSON, K. H., Hydrothermal stability and intermolecular organization of collagens from mammalian and teleost skins. *Svensk kem. Tidskr.* 1953. *65*. 70—76.

GUSTAVSON, K. H., Hydroxyproline and stability of collagens. *Acta chem. scand.* 1954a. *8*. 1298—1299.

GUSTAVSON, K. H., The presence of interchain links between hydroxy and keto-imide groups in collagen. *Acta chem. scand.* 1954b. *8*. 1299—1301.

GUSTAVSON, K. H., The function of hydroxyproline in collagens. *Nature* (Lond.) 1955. *175*. 70—74.

GUSTAVSON, K. H., *The Chemistry and Reactivity of Collagen.* New York, Academic Press 1956. pp. 342.

HAFTER, R. und H. HÖRMANN, Der Einfluss von Pepsin auf die Struktur und die faserbildenden Eigenschaften von Kollagen. *Hoppe-Seylers Z. physiol. Chem.* 1963. *330*. 169—181.

HALL, C. E. and P. DOTY, A comparison between the dimensions of some macromolecules determined by electron microscopy and by physical chemical methods. *J. Amer. chem. Soc.* 1958. *80*. 1269—1274.

HALL, D. A. (ed.), *International Review of Connective Tissue Research.* New York, Academic Press 1963. *1*. pp. 401., 1964. *2*. pp. 350., 1965. *3*. pp. 281.

HALL, D. A. and R. REED, Hydroxyproline and thermal stability of collagen. *Nature* (Lond.) 1957. *180*. 243.

HAMER, D. and J. MARCHANT, Collagen and other constituents in the skin of normal, carcinogen-treated and castrated mice. *Brit. J. Cancer* 1957. *11*. 445—451.

HAMILTON, P. B., Ion exchange chromatography of amino acids. Effect of resin particle size on column performance. *Analyt. Chem.* 1958. *30*. 914—919.

HAMILTON, P. B. and R. A. ANDERSON, Demonstration of ornithine in gelatin by ion exchange chromatography. *J. biol. Chem.* 1954. *211*. 95—102.

HANNIG, K. and J. ENGEL, Physikalisch-chemische Untersuchungen an Tropokollagen lösungen. *Leder* 1961. *12*. 213—222.

HARDING, J. J., The components of gelatins derived from soluble collagens. *J. Soc. Leather Tr. Chem.* 1964. *48*. 160—173.

HARDING, J. J., The unusual links and cross-links of collagen. In: Anfinsen, Jr., C. B., M. L. Anson, J. T. Edsall and F. M. Richards (eds.), *Advances in Protein Chemistry.* New York, Academic Press 1965. *20*. 109—190.

HARKNESS, M. L. R., R. D. HARKNESS and D. W. JAMES, The effect of a protein-free diet on the collagen content of mice. *J. Physiol.* (Lond.) 1958. *144*. 307—313.

HARKNESS, R. D., Biological functions of collagen. *Biol. Rev.* 1961. *36*. 399—463.

HARRINGTON, W. F., Effect of neutral salts on the structure of collagen and gelatin. *Nature* (Lond.) 1958. *181*. 997—998.

HARRINGTON, W. F., On the arrangement of the hydrogen bonds in the structure of collagen. *J. molec. Biol.* 1964. *9*. 613—617.

HARRINGTON, W. F. and P. H. VON HIPPEL, The structure of collagen and gelatin. In: Anfinsen, Jr., C. B., M. L. Anson, K. Bailey and J. T. Edsall (eds.), *Advances in Protein Chemistry.* New York, Academic Press 1961. *16*. 1—138.

HARRINGTON, W. F. and O. W. MCBRIDE, The stabilization of collagen. *Birth Defects Original Article Series* 1966. *2*. 10—17.

HARRIS, Jr., E. and A. SJOERDSMA, Collagen content, solubility, and cross-linking in human dermis. *J. clin. Invest.* 1966. *45*. 1020.

HEIDRICH, H-G. and L. K. WYNSTON, A report of the presence of a third α-component in calf and rat skin tropocollagens. *Hoppe-Seylers Z. physiol. Chem.* 1965. *342*. 166—169.

HEIKKINEN, E. and E. KULONEN, Age factor in the maturation of collagen. Intramolecular linkages in mildly denatured collagen. *Experientia* 1964. *20*. 310.

VON HIPPEL, P. H. and K-Y. WONG, The collagen $\rightleftarrows$ gelatin phase transition. I. Further studies of the effects of solvent environment and polypeptide chain composition. *Biochemistry* 1963a *2*. 1387—1398.

VON HIPPEL, P. H. and K-Y. WONG, The collagen $\rightleftarrows$ gelatin phase transition. II Shape of the melting curves and effect of chain length. *Biochemistry* 1963b. *2*. 1399—1413.

HIRS, C. H. W., W. H. STEIN and S. MOORE, The amino acid composition of ribonuclease. *J. biol. Chem.* 1954. *211*. 941—950.

HOLZMANN, H., G. W. KORTING, F. HAMMERSTEIN, K. STECHER, M. DURRUTI, P. IWANGOFF and K. KÜHN, Quantitative Bestimmungen der einzelnen Kollagenfraktionen der Haut nach Anwendung von Resochin und Progesteron. *Naturwissenschaften* 1964. *51*. 310.

HÖRMANN, H., Zur Frage der Quervernetzung von Kollagen. *Leder* 1962. *13*. 79—86.

INGRAM, V. M., Abnormal human haemoglobins I. The comparison of normal human and sickle-cell haemoglobins by "fingerprinting". *Biochim. biophys. Acta* (Amst.) 1958. *28*. 539—545.

INGRAM, V. M., Gene evolution and the haemoglobins. *Nature* (Lond.) 1961. *189*. 704—708.

ISAACS, W. A., K. LITTLE, J. D. CURREY and L. B. H. TARLO, Collagen and a cellulose-like substance in fossil dentine and bone. *Nature* (Lond.) 1963. *197*. 192.

JACKSON, D. S., A. A. LEACH and S. JACOBS, The amino acid composition of the collagen fractions of rabbit skin. *Biochim. biophys. Acta* (Amst.) 1958. *27*. 418—420.

JACKSON, D. S. and A. NEUBERGER, Observations on the isoionic and isoelectric point of acid-processed gelatin from insoluble and citrate-extracted collagen. *Biochim. biophys. Acta* (Amst.) 1957. *26*. 638—639.

JACKSON, S. F., R. D. HARKNESS, S. M. PARTRIDGE and G. R. TRISTRAM (eds.), *Structure and Function of Connective and Skeletal Tissue*. London, Butterworths 1965. pp. 537.

JOSEPH, K. T., K. R. RAJENDRAN, V. MOHANARADHAKRISHNAN, N. RAMANATHAN and Y. NAYUDAMMA, Studies on dugong hide collagen. *J. Amer. Leather chem. Assoc.* 1964. *59*. 462—474.

JOSSE, J. and W. F. HARRINGTON, Role of pyrrolidine residues in the structure and stabilization of collagen. *J. molec. Biol.* 1964. *9*. 269—287.

KAO, K-Y. T., D. M. HILKER and T. H. MCGAVACK, Connective tissue III. Collagen and hexosamine content of tissues of rats of different ages. *Proc. Soc. exp. Biol.* (N.Y.) 1960. *104*. 359—361.

KAWAI, S., S. HASHIMOTO, T. SODE, T. MIYATA and T. NISHIHARA, The detailed structural aspects on neutral salt soluble and acid soluble collagen macromolecules. In: Comte, P. (ed.), *Biochimie et Physiologie du Tissu Conjonctif*. Lyon 1966. 249—258.

KESSLER, A., H. ROSEN and S. M. LEVENSON, Chromatographic fractionation of acetic acid-solubilized rat tail tendon collagen. *J. biol. Chem.* 1960. *235*. 989—994.

KORTING, G. W., H. HOLZMANN und K. KÜHN, Biochemische Bindegewebsanalysen bei progressiver Sklerodermie. *Klin. Wschr.* 1964. *42*. 247—248.

KULONEN, E., T. HOLLMÉN, V. NÄNTÖ and J. PIKKARAINEN, Experience on the starch gel electrophoresis of heat-denatured collagens. In: Jackson, S. F., R. D. Harkness,

S. M. Partridge and G. R. Tristram (eds.), *Structure and Function of Connective and Skeletal Tissue*. London, Butterworths 1965. 216—221.

KÜHN, K., Investigations of the renaturation of tropocollagen. In: Ramachandran, G. N. (ed.), *Aspects of Protein Structure*. London, Academic Press 1963. 279—287.

KÜHN, K., M. DURRUTI, P. IWANGOFF, F. HAMMERSTEIN, K. STECHER, H. HOLZMANN and G. W. KORTING, Untersuchungen über den Stoffwechsel des Kollagens, I. Der Einbau von (^{14}C)Glycin in Kollagen bei lathyritischen Ratten. *Hoppe-Seylers Z. physiol. Chem.* 1964a. *336*. 4—12.

KÜHN, K., P. FIETZEK and J. KÜHN, The action of proteolytic enzymes on collagen. *Biochem. Z.* 1966. *344*. 418—434.

KÜHN, K., P. IWANGOFF, F. HAMMERSTEIN, K. STECHER, M. DURRUTI, H. HOLZMANN and G. W. KORTING, Untersuchungen über den Stoffwechsel des Kollagens, II. Der Einbau von (^{14}C)Glycin in Kollagen bei mit Prednison behandelten Ratten. *Hoppe-Seylers Z. physiol. Chem.* 1964b. *337*. 249—256.

KÜNTZEL, A., Über einige Versuche der Rekonstitution von Kollagenfasergeflechten mit Hilfe löslichen Kollagens. *Leder* 1964. *15*. 248—249.

LEACH, A. A., The amino acid composition of amphibian, reptile and avian gelatins. *Biochem. J.* 1957. *67*. 83—87.

LEVENE, C. I. and J. GROSS, Alterations in state of molecular aggregation of collagen induced in chick embryos by β-aminopropionitrile (lathyrus factor). *J. exp. Med.* 1959. *110*. 771—790.

LEWIS, M. S. and K. A. PIEZ, Physical properties of dogfish shark skin collagen. *Fed. Proc.* 1961. *20*. 380.

LEWIS, M. S. and K. A. PIEZ, The characterization of collagen from the skin of the dogfish shark, *Squalus acanthias*. *J. biol. Chem.* 1964. *239*. 3336—3340.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with the Folin phenol reagent. *J. biol. Chem.* 1951. *193*. 265—275.

MAJEWSKI, C., Z. LEJA and H. MAJEWSKA, Studies on healthy collagen in the skin of persons deceased from diseases of the internal organs. *Pat. pol.* 1964. *15*. 1—5.

MANSKI, W., S. P. HALBERT and T. P. AUERBACH, Immunochemical analysis of the phylogeny of lens proteins. In: Leone, C. A. (ed.), *Taxonomic Biochemistry and Serology*. New York, The Ronald Press Co. 1964. 545—554.

MARGOLIASH, E., Primary structure and evolution of cytochrome *c*. *Proc. nat. Acad. Sci.* (Wash.) 1963. *50*. 672—679.

MARGOLIASH, E. and E. L. SMITH, Structural and functional aspects of cytochrome *c* in relation to evolution. In: Bryson, V. and H. J. Vogel (eds.), *Evolving Genes and Proteins*. New York, Academic Press 1965. 221—242.

MASER, M. D. and R. V. RICE, Biophysical and biochemical properties of earthworm-cuticle collagen. *Biochim. biophys. Acta* (Amst.) 1962, *63*. 255—265.

MASER, M. D. and R. V. RICE, Soluble earthworm cuticle collagen: a possible dimer of tropocollagen. *J. cell. Biol.* 1963a. *18*. 569—577.

MASER, M. D. and R. V RICE, The denaturation and renaturation of earthworm-cuticle collagen. *Biochim. biophys. Acta* (Amst.) 1963b. *74*. 283—294.

MATHEWS, M. B., E. KULONEN and A. DORFMAN, Studies in procollagen. II. Viscosity and molecular weight. *Arch. Biochem.* 1954. *52*. 247—260.

MAYR, *E.*, The new systematics. In: Leone, C. A. (ed.), *Taxonomic Biochemistry and Serology*. New York, The Ronald Press Co. 1964. 13—32.

MCGAVACK, T. H. and K-Y. T. KAO, The influence of age and sex on the soluble

collagen, insoluble collagen and elastin of rat tissue. *Exp. Med. Surg.* 1960. *18.* 104—123.

MILLS, B. G. and L. A. BAVETTA, Variations in the extractable collagen of bone and skin with age and growth. *J. Geront.* 1966. *21.* 449—454.

MINARI, O. and D. B. ZILVERSMIT, Use of KCN for stabilization of color in direct nesslerization of Kjeldahl digests. *Analyt. Biochem.* 1963. *6.* 320—327.

MOORE, S., D. H. SPACKMAN and W. H. STEIN, Chromatography of amino acids on sulfonated polystyrene resins. An improved system. *Analyt. Chem.* 1958. *30.* 1185—1190.

MURRAY, D. H., W. R. WATTS and J. R. RING, Hexosamine and hydroxyproline concentrations in skin and buccal mucosa of an aging rat population. *J. Geront.* 1961. *16.* 17—19.

MURRAY, K., P. S. RASMUSSEN, J. NEUSTAEDTER and J. M. LUCK, The hydrolysis of arginine. *J. biol. Chem.* 1965. *240.* 705—709.

NIMNI, M. E. and L. A. BAVETTA, Collagen synthesis and turnover in the growing rat under the influence of methyl prednisolone. *Proc. Soc. exp. Biol.* (N.Y.) 1964. *117.* 618—623.

NIMNI, E., E. DEGUIA and L. A. BAVETTA, Collagen, hexosamine and tensile strength of rabbit skin during aging. *J. invest. Derm.* 1966. *47.* 156—158.

NORDWIG, A. and U. HAYDUK, A contribution to the evolution of collagen. *J. molec. Biol.* 1967. *26.* 351—352.

NÄNTÖ, V., J. MAATELA and E. KULONEN, Separation of the subunits of denatured collagens and gelatins with starch gel electrophoresis. *Acta chem. scand.* 1963. *17.* 1604—1608.

NÄNTÖ, V., J. PIKKARAINEN and E. KULONEN, Optimal conditions in the starch gel electrophoresis of heat-denatured collagen. *J. Amer. Leather chem. Ass.* 1965. *60.* 63—71.

OLSEN, B. R., Electron microscope studies on collagen. IV. Structure of vitrosin fibrils and interaction properties of vitrosin molecules. *J. Ultrastruct. Res.* 1965. *13.* 172—191.

OREKHOVITCH, V. N. and V. O. SHPIKITER, Physicochemical nature of procollagen. In: Stainsby, G. (ed.), *Recent Advances in Gelatin and Glue Research.* London, Pergamon Press 1958a. 87—91.

OREKHOVITCH, V. N. and V. O. SHPIKITER, Procollagens. *Science* 1958b. *127.* 1371—1376.

OREKHOVITCH, V. N., V. O. SHPIKITER, V. I. MAZUROV and V. O. KOUNINA, Procollagènes — classification, métabolisme, action des protéinases. *Bull. Soc. chim. biol.* (Paris) 1960. *42.* 505—518.

PAGE, I. H., *Connective Tissue, Thrombosis and Atherosclerosis.* New York, Academic Press 1959. pp. 316.

PIEZ, K. A., Nonidentity of the three α chains in codfish skin collagen. *J. biol. Chem.* 1964. *239.* PC4315—PC4316.

PIEZ, K. A., Characterization of a collagen from codfish skin containing three chromatographically different α chains. *Biochemistry* 1965. *4.* 2590—2596.

PIEZ, K. A. and A. L. CARRILLO, Helix formation by single- and double-chain gelatins from rat skin collagen. *Biochemistry* 1964. *3.* 908—914.

PIEZ, K. A., E. A. EIGNER and M. S. LEWIS, The chromatographic separation and amino acid composition of the subunits of several collagens. *Biochemistry* 1963. *2.* 58—66.

PIEZ, K. A. and J. GROSS, The amino acid composition of some fish collagens: The relation between composition and structure. *J. biol. Chem.* 1960. *235*. 995—998.

PIEZ, K. A., M. S. LEWIS, G. R. MARTIN and J. GROSS, Subunits of the collagen molecule. *Biochim. biophys. Acta* (Amst.) 1961. *53*. 596—598.

PIEZ, K. A. and R. C. LIKINS, The nature of collagen. II. Vertebrate collagens. In: Sognnaes, R. F. (ed.), *Calcification in Biological Systems*. Washington, American Association for the Advancement of Science. 1960. 411—420.

PIEZ, K. A., E. WEISS and M. S. LEWIS, The separation and characterization of the α- and β-components of calf skin collagen. *J. biol. Chem.* 1960. *235*. 1987—1991.

PIKKARAINEN, J. and E. KULONEN, Electrophoretic fractionation of the various subunits from heat-denatured insoluble collagen. *Acta chem. scand.* 1964. *18*. 2011—2012.

PIKKARAINEN, J. and E. KULONEN, Collagen of lamprey. *Acta chem. scand.* 1965. *19*. 280.

PIKKARAINEN, J. and E. KULONEN, Preservation of native collagen. *Suomen Kemistilehti B* 1966. *39*. 156—157.

PIKKARAINEN, J. and E. KULONEN, On the evolution and development of collagen. *Scand. J. clin. Lab. Invest.* 1967. Suppl. 95. 40.

PIKKARAINEN, J., J. RANTANEN and E. KULONEN, On the comparative biochemistry of collagen. In: Comte, P. (ed.), *Biochimie et Physiologie du Tissu Conjonctif*. Lyon 1966. 303—312.

POLATNICK, J., A. J. LA TESSA and H. M. KATZIN, Comparison of collagen preparations from beef cornea and sclera. *Biochim. biophys. Acta* (Amst.) 1957. *26*. 365—369.

RAO, N. V. and W. F. HARRINGTON, Relationship between the positioning of pyrrolidine residues and the stability of collagen. *J. molec. Biol.* 1966. *21*. 577—581.

REICH, G., Untersuchungen über Kollagen und Gelatine X. Über die elektrophoretische Trennung der Denaturierungsprodukte des Tropokollagens an Polyacrylamidgelen. *Ges. Abh. DLI* 1964. *19*. 123—129.

REICH, G., *Kollagen*. Dresden, Verlag Theodor Steinkopff 1966. pp. 308.

RICE, R. V., E. F. CASASSA, R. E. KERWIN and M. D. MASER, On the length and molecular weight of tropocollagen from calf skin. *Arch. Biochem.* 1964. *105*. 409—423.

RICH, A. and F. H. C. CRICK, The molecular structure of collagen. *J. molec. Biol.* 1961. *3*. 483—506.

RIESS, W., Ausbeutesteigerung beim Auflösen von Kollagen durch verdünnte Säuren. *Leder* 1964. *15*. 233—237.

RIGBY, B. J., Two-stage contraction in rat-tail tendon. *Biochim. biophys. Acta* (Amst.) 1961. *47*. 534—538.

RIGBY, B. J., Thermal transitions in normal and deuterated rat-tail tendon, human skin, and tuna-fish skin. *Biochim. biophys. Acta* (Amst.) 1962. *62*. 183—185.

RIGBY, B. J., Relation between the shrinkage of native collagen in acid solution and the melting temperature of the tropocollagen molecule. *Biochim. biophys. Acta* (Amst.) 1967. *133*. 272—277.

ROGULENKOVA, V. N., M. I. MILLIONOVA and N. S. ANDREEVA, On the close structural similarity between poly-Gly-L-Pro-L-Hypro and collagen. *J. molec. Biol.* 1964. *9*. 253—254.

RUBIN, A. L., M. P. DRAKE, P. F. DAVISON, D. PFAHL, P. T. SPEAKMAN and F. O. SCHMITT, Effects of pepsin treatment on the interaction properties of tropocollagen macromolecules. *Biochemistry* 1965. *4*. 181—190.

SAKATA, R., Biochemical studies on the collagen metabolism I. Collagen metabolism in normal skin. *Kumamoto med. J.* 1960. *13*. 27—40.

SCHLEYER, M., Aminosäuresequenzen des Kollagens, V. Trennung der α- und β-Komponenten aus Tropokollagen. *Hoppe-Seylers Z. physiol. Chem.* 1962. *329*. 97—104.

SCHLUETER, R. J. and A. VEIS, The macromolecular organization of dentine matrix collagen. II. Periodate degradation and carbohydrate cross-linking. *Biochemistry* 1964. *3*. 1657—1665.

SIEGEL, F. L. and M. K. ROACH, β-2-Thienyl-DL-alanine, internal standard for automatic determination of amino acids. *Analyt. Chem.* 1961. *33*. 1628.

SMITS, G., J. MORREAU, C. GARCIA-SMIT and S. STODEL, Quantitative interrelationships of the chief components of some connective tissues during foetal and post-natal development in cattle. *Biochim. biophys. Acta* (Amst.) 1957. *25*. 542—548.

SOBEL, H., S. GABAY, E. T. WRIGHT, I. LICHTENSTEIN and N. H. NELSON, The influence of age upon the hexosamine-collagen ratio of dermal biopsies from men. *J. Geront.* 1958. *13*. 128—131.

SPACKMAN, D. H., W. H. STEIN and S. MOORE, Automatic recording apparatus for use in the chromatography of amino acids. *Analyt. Chem.* 1958. *30*. 1190—1206.

STAINSBY, G. (ed.), *Recent Advances in Gelatin and Glue Research*. London, Pergamon Press 1958. pp. 277.

STEGEMANN, H., Mikrobestimmung von Hydroxyprolin mit Chloramin-T und p-dimethylaminobenzaldehyd. *Hoppe-Seylers Z. physiol. Chem.* 1958. *311*. 41—45.

STEVEN, F. S. and G. R. TRISTRAM, The denaturation of acetic acid-soluble calf-skin collagen. Changes in optical rotation, viscosity and susceptibility towards enzymes during serial denaturation in solutions of urea. *Biochem. J.* 1962. *85*. 207—210.

SZENT-GYÖRGYI, A. G. and C. COHEN, Role of proline in polypeptide chain configuration of proteins. *Science* 1957. *126*. 697—698.

SZENTIRMAI, A., P. BRAUN, I. HORVÁTH and M. HAUK, A rapid screening test for determination of total α-amino acids in urine and serum. *Clin. chim. Acta* 1962. *7*. 459—462.

TAKAHASHI, T. and T. TANAKA, Physico-chemical studies on the skin and leather of marine animals. VIII. On the heat stability of fish skin and leather. *Bull. Jap. Soc. Sci. Fisheries* 1953. *19*. 603—610.

TANZER, M. L., D. MONROE and J. GROSS, Inhibition of collagen intermolecular cross-linking by thiosemicarbazide. *Biochemistry* 1966. *5*. 1919—1926.

TRAUB, W. and A. YONATH, Polymers of tripeptides as collagen models I. X-Ray studies of Poly(L-prolyl-glycyl-L-proline) and related polytripeptides. *J. molec. Biol.* 1966. *16*. 404—414.

URNES, P. and P. DOTY, Optical rotation and the conformation of polypeptides and proteins. In: Anfinsen, Jr., C. B., M. L. Anson, K. Bailey and J. T. Edsall (eds.), *Advances in Protein Chemistry*. New York, Academic Press 1961. *16*. 401—544.

WALSH, K. A. and J. R. BROWN, Internal standards for amino acid analyses. *Biochim. biophys. Acta* (Amst.) 1962. *58*. 596—598.

WARD, A. G., Conversion of collagen to gelatin and chemical composition. In: Stainsby, G. (ed.), *Recent Advances in Gelatin and Glue Research*. London, Pergamon Press 1958. 137—139.

VEIS, A., *The Macromolecular Chemistry of Gelatin*. New York, Academic Press 1964. pp. 433.

VEIS, A. and J. ANESEY, Modes of intermolecular cross-linking in mature insoluble collagen. *J. biol. Chem.* 1965. *240*. 3899—3908.

VEIS, A., J. ANESEY and J. COHEN, The depolymerization of collagen fibers. *J. Amer. Leather chem. Ass.* 1960. *55*. 548—563.

VEIS, A., J. ANESEY and J. COHEN, The characterization of the γ-component of gelatin. *Arch. Biochem.* 1962. *98*. 104—110.

VEIS, A. and M. P. DRAKE, The introduction of intermolecular covalent cross-linkages into ichthyocol tropocollagen with monofunctional aldehydes. *J. biol. Chem.* 1963. *238*. 2003—2011.

VEIS, A. and R. J. SCHLUETER, The macromolecular organization of dentine matrix collagen. I. Characterization of dentine collagen. *Biochemistry* 1964. *3*. 1650—1657.

VERZAR, F., Nachweis der Zunahme der Bindung von Hydroxyprolin im Kollagen der Haut mit dem Alter. *Gerontologia* (Basel) 1960. *4*. 104—111.

VERZAR, F., Factors which influence the age-reaction of collagen in the skin. *Gerontologia* (Basel) 1964. *9*. 209—221.

WIRTSCHAFTER, Z. T. and J. P. BENTLEY, The influence of age and growth rate on the extractable collagen of skin of normal rats. *Lab. Invest.* 1962. *11*. 316—320.

WOESSNER, JR., J. F., The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch. Biochem.* 1961. *93*. 440—447.

YANG, J. T. and P. DOTY, The optical rotatory dispersion of polypeptides and proteins in relation to configuration. *J. Amer. chem. Soc.* 1957. *79*. 761—775.

YOUNG, E. G. and J. W. LORIMER, The acid-soluble collagen of cod skin. *Arch. Biochem.* 1960. *88*. 373—381.

YOUNG, E. G. and J. W. LORIMER, A comparison of the acid-soluble collagens from the skin and swim bladder of the cod. *Arch. Biochem.* 1961. *92*. 183—190.

ZONTA, A. and M. CAMPANI, La componente fibrillare del tessuto connettivo. *Biochim. biol. Sper.* 1965. *4*. 82—130.

ZUCKERKANDL, E. and L. PAULING, Evolutionary divergence and convergence in proteins. In: Bryson, V. and H. J. Vogel (eds.), *Evolving Genes and Proteins*. New York, Academic Press 1965. 97—166.